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Author(s)	Fukumori, Yoshihiro
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UNIVERSITY OF OSAKA

OSAKA

Studies on Oxidation of Inorganic Sulfur Compounds
in <u>Chromatium vinosum</u>

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Physiology

by

Yoshihiro Fukumori

Committee in charge:

Professor Hiroshi Matsubara

Professor Yuji Tonomura

Assistant Professor Tateo Yamanaka

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CONTENTS

STUDIES ON OXIDATION OF INORGANIC SULFUR COMPOUNDS
IN CHROMATIUM VINOSUM

ABBREVIATIONS

SDS   Sodium dodecyl sulfate

PTH   Phenylthiohydantoin derivative

EDTA   Ethylenediamine tetraacetic acid

FAD   Flavine-Adenine dinucleotide

FMN   Flavine mononucleotide

DEAE-cellulose   Diethylamino ethyl cellulose

CO   Carbon monoxide

Em,7   Midpoint potential at pH 7.0

DCIP   2,6-Dichlorophenolindophenol

MV   Methylviologen

ε_{mM} Millimolar absorption extinction coefficient

NAD   Nicotinamide adenine dinucleotide

ORD   Optical rotatory dispersion

Tris   Tris(hydroxymethyl) aminomethane

(III)

ACKNOWLEDGEMENTS

PUBLICATIONS

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  "Thibacillus novellus cytochrome oxidase can separate
  some eukaryotic cytochromes"
    FEBS letters (1977) 77(2), 155-158

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  "Cytochrome c-554 derived from the blue-green alga
  Spirulina platensis"
    Plant and Cell Physiology (1978) 19(1), 117-126

T.Yamanaka, & Y.Fukumori

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  with cytochrome oxidase"
    in "Evolution of Protein Molecules"(H.Matsubara, & T.Yamanaka
    eds.) pp.387-399,(1978), Japan Scientific Societies Press,
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  Chromatium vinosum"
    Analytical Biochemistry (1979) 95(1), 209-213

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  "A HiPIP-Linked Thiosulfate-Oxidizing Enzyme Derived from
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    Current Microbiology (1980) 3 in press

ABSTRACT

Although it has been known that the purple sulfur bacterium, Chromatium vinosum, acquires energy and reducing power by photosynthetic oxidation of inorganic sulfur compounds such as thiosulfate and sulfide, little was known about the oxidation mechanisms of the sulfur compounds in the organism. From C.vinosum a flavocytochrome c, cytochrome c-552 has been isolated. The cytochrome possesses two molecules of heme c and one molecule of covalently-bound FAD per molecule. Although the physical and chemical properties of the cytochrome have been clarified to some extent, its function was unknown.

In Part I, the function and structural features of Chromatium cytochrome c-552 have been investigated. Cytochrome c-552 has a sulfide-cytochrome c reductase activity and also catalyzes reduction of elementary sulfur to sulfide with reduced benzylviologen as the electron donor. In the sulfide-cytochrome reduction, horse cytochrome c and yeast cytochrome c act as good electron acceptors, but cytochrome c' or cytochrome c-553(550) purified from the organism does not. Further, it has become clear that cytochrome c-552 is much more rapidly reduced with sulfide than other cytochromes c such as horse cytochrome c and that electrons are sequentially transported in the cytochrome molecule from flavin to heme c in the oxidation of sulfide.

The subunit structure of cytochrome c-552 has been studied. The cytochrome is split by 6 M urea into cytochrome and flavoprotein moieties with molecular weights of 21,000 and 46,000, respectively. The flavoprotein moiety is obtained by isoelectric focusing in the

presence of 6 M urea and 0.1 % β-mercaptoethanol, while the hemo-protein moiety is obtained by gel filtration with Sephacryl S-200 in the presence of 6 M urea and 0.1 M KCl. Neither of the subunits has the sulfide-cytochrome c reductase activity. Attempts to re-constitute the original flavocytochrome c from the subunits have been unsuccessful.

In Part II, the reactions between reduced Chromatium cyto-chrome c-552 and carbon monoxide have been studied by stopped-flow and flash-photolysis. It has been found that two hemes in cytochrome c-552 reacts with carbon monoxide with different affinities and CO-cytochrome c-552 is fairly light senstive.

In Part III, some enzymatic properties of a thiosulfate-oxidizing enzyme have been studied. The enzyme, partially purified from Chromatium vinosum, reduces rapidly HiPIP (high potential nonheme iron protein) in the presence of thiosulfate. Cytochromes c of yeast and tuna, and ferricyanide act also as good electron acceptors. Cytochrome c-552, cytochrome c' or cytochrome c-553(550) does not act as electron acceptor. The enzyme is inhibited by cyanide and sulfite. On the basis of the stoichiometry in reduction of ferricyanide catalyzed by the enzyme in the presence of thio-sulfate, the oxidized product of thiosulfate is inferred to be tetrathionate.

I.   STRUCTURE AND FUNCTION OF FLAVOCYTOCHROME C

DERIVED FROM CHROMATIUM VINOSUM

INTRODUCTION

Photosynthetic bacteria are classified into three groups; green sulfur bacteria, purple sulfur bacteria and non-sulfur purple bacteria. Among these bacteria, purple and green sulfur bacteria obtain energy and reducing power by photosynthetic oxidation of thiosulfate and sulfide (1). These organisms inhabit lakes and swamps, where the sulfate-reducing bacteria produce sulfide in the mud (2).

Van Niel has demonstrated that oxidation of sulfide by Chromatiaceae (purple sulfur bacteria) and Chlorobiaceae (green sulfur bacteria) is in stoichiometrical relationship with fixation of carbon dioxide;

$$2H_2S \ + \ CO_2 \ \longrightarrow \ 2S \ + \ H_2O \ + \ (CH_2O)$$
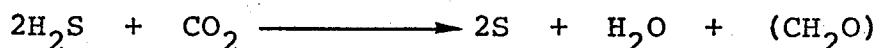
Various workers later studied the electron transfer pathways in the chromatophore of Chromatium vinosum. Olson et al. (3,4,50) have suggested that cytochromes c-552, c-553 and c' in Chromatium are involved in two different electron-transfer pathways and that cytochrome c-552 may function in the non-cyclic electron transport chain as a link between inorganic electron donors (S^{2-}, S, $S_2O_3^{2-}$) and the main photosynthetic electron transport system. This suggestion was supported by Morita et al. (5). These authors have found that cytochrome c-552 in anaerobically starved cells is in the oxidized state but is reduced on addition of H_2, S^{2-} and $S_2O_3^{2-}$. More recently, evidence has been presented by Cusanovitch et al. (6) that high-potential cytochrome c-553 and low-potential cytochrome c-552 appear to be oxidized by different reaction centers and each reaction

center is associated with a spectrally different array of light harvesting chlorophyll (7). Takamiya et al. have suggested the possibility of the presence of two types of association between the cytochromes and reaction-center bacteriochlorophyll (8).

On the other hand, Parson (9) and Thronber (10) have argued that both the cytochromes are oxidized by the same photosystem. This system has been studied in some detail with C.vinosum; it is the cyclic electron transport system which is driven by the P-883-P-800 reaction center complex. Tiede et al. studied the magnetic properties of the cytochrome and bacteriochlorophyll of the reaction center in C.vinosum and proposed a structural organization of the C.vinosum reaction center and the associated cytochromes c (11).

Several kinds of C-type cytochrome have been purified from Chromatium vinosum; cytochrome c-552 (12-16), cytochrome c-553 (17), cytochrome c'(formerly called cytochrome cc') (12) and a cholate-solubilized cytochrome complex (18). Chromatium cytochrome c-552 possesses two molecules of heme c and one molecule of covalently-bound FAD per molecule (13,19-21). Although the physical and chemical properties of the cytochrome have been clarified to some extent, its function was unknown. Flavocytochrome c have been found in two genera of photosynthetic sulfur bacteria, Chromatium and Chlorobium (13). In these organisms sulfide is known to be oxidized to sulfate via elementary sulfur (22,23).

In the present investigation, the author has found the purified cytochrome c-552 has a sulfide-cytochrome c reductase

activity and that electrons are sequentially transported in the
cytochrome molecule from flavin to heme c in the oxidation of
sulfide. Further, it has been found that cytochrome c-552 cata-
lyzes also reduction of elementary sulfur with an appropriate
electron donor; e.g. with reduced benzylviologen. This suggests
that the cytochrome may also participate in reduction of ele-
mentary sulfur to sulfide in vivo, as whole cells of C.vinosum
(27) and C.limicola f. thiosulfatophilum (25) have been known
to be catalyzed reduction of elementary sulfur to sulfide under
some conditions.

It is interesting that C.vinosum cytochrome c-552 possesses
two molecules of heme c and one molecule of FAD in one molecule
(13),while the counterpart in C.limicola f. thiosulfatophilum,
cytochrome c-553 has one molecule each of heme c and flavin
in one molecule (13). We have succeeded in unveiling to some
extent the structural features of cytochrome c-552; the cyto-
chrome molecule is split into two subunits with molecular weight
of 46,000 and 21,000. The larger subunit contains flavin, and
the smaller one heme. Some properties of each subunit have been
studied.

MATERIALS AND METHODS

Cultivation of the Organism

A strain of Chromatium vinosum was kindly supplied by
Drs.R.G.Bartsch and T.Meyer (University of California, San Diego,
U.S.A.) and large-scale culture of the organism was performed
as described by Bartsch and Kamen (12). The cultivation medium

contained in 1 liter: 10.0g NaCl, 0.5g K_2HPO_4, 0.5g KH_2PO_4, 1.0g NH_4Cl, 0.5g $MgCl_2 6H_2O$, 0.05g $CaCl_2$, 2.0g $NaHCO_3$, 0.005g $FeCl_3 6H_2O$, 2.0g $Na_2S_2O_3 5H_2O$, 1.0g $Na_2S 9H_2O$. Deionized water was used to dissolve these reagents. The pH of the medium was adjusted to 8.0 with H_3PO_4. About 20 glass bottles (10 liters in volume) were completely filled with medium and stoppered with Parafilm. The medium in each bottle was inoculated with 200 ml of the seed culture of the organism, and the bottles were illuminated by 100 watt incandescent lamps from a distance of 30 cm at about 30°C. Cells were harvested after growth for 5 days and stored at -20°C before use.

Reagents

Cytochrome c-552 and cytochrome c' were purified by the methods of Bartsch and Kamen (12,13) and of Bartsch (12), respectively, with slight modifications, and cytochrome c-553(550) by the method of Cusanovich and Bartsch (17). Cytochrome c (555, C.limicola f. thiosulfatophilum) (28), cytochrome c (552, Nitrosomonas europaea) (29), and cytochrome c (554, Pseudomonas aeruginosa) (30) were purified by the methods previously established in our laboratory, and cytochrome c_2 (Rhodospirillum rubrum) was kindly supplied by Dr.Horio (Institute for Protein Research, Osaka University). Horse cytochrome c (type VI) was purchased from Sigma Chemical Co., U.S.A.. Elementary sulfur was prepared by the method of Roy and Trudinger (31). Various chemicals were reagent grade and purchased from Wako Pure Chemical Industries, Ltd. (Osaka) and Nakarai Chemicals, Ltd. (Kyoto).

Spectrophotometric Determinations

Absorption spectra at room temperature were determined in a Cary recording spectrophotometer, model 15 or 16. The reduction of cytochrome c with Na_2S in the presence of cytochrome c-552 was followed spectrophotometrically in terms of the increase of the absorbance at the α-peak of each cytochrome. The reduction of elementary sulfur with reduced benzylviologen was performed anaerobically using a Thunberg-type cuvette, and followed in terms of the decrease of the absorbance at 550 nm.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate

Polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Weber and Osborn (32) with slight modifications; a slab gel, 14.3 x 10 x 0.1 cm, was made by polymelization of 7.5 % acrylamide and soaked overnight in 0.1 M Tris-HCl buffer, pH 8.5 containing 0.5 % SDS, 6M urea, and 1 % β-mercaptoethanol. After electrophoresis had been performed for 1.5 h, the gel was stained with Coomassie brilliant blue R-250 in a mixture of 30 % methanol and 10 % trichloroacetic acid and then decolorized with a mixture of 7 % acetic acid and 30 % methanol.

Isoelectric Point Determination

Isoelectric point was determined by isoelectric focusing as described by Vesterberg and Svensson (34), using an apparatus with a volume of 110 ml and carrier ampholyte (LKB, Sweden) in the pH range 3-6 or 3-10. The temperature of the column was maintained at $4^{\circ}C$ and the electric power at about 1.8 watts.

Electrophoresis was performed for about two days.

Analysis of the Amino Terminal Sequences

The amino terminal sequences were determined by the Edman degradation procedure (35). Identification of the PTH-derivatives of amino acids was carried out by thin-layer chromatography (36).

Determination of Amino Acids Composition

The sample was hydrolyzed with 6 N HCl for 24 h or 72 h at $110^{\circ}C$ in a sealed tube after evacuation. The hydrolysate thus obtained was dried and analyzed in a Beckman-Spinco amino acid analyzer, model 120B, with an acceleration system according to the method of Spackman et al. (37). The amount of cysteine was determined with a performic acid-oxidized sample (38), and that of the cysteine residue bound to heme by the method of Fontana et al. (39).

Analysis of Sulfide

To 3.0 ml of the reaction mixture which contained sulfide, was added a mixture of 0.5 ml each of Na_2CO_3 (10 %) and $ZnSO_4 7H_2O$ (20 %). The resulting suspension was stirred for 1 min and centrifuged at 9,000 g for 10 min. To the precipitate thus collected was added 1.0 ml of 6N HCl, and H_2S gas generated was trapped in 1.0 ml of 0.1 N NaOH. The alkaline solution thus obtained was analyzed by the methylene blue method (40).

Measurements of Oxidation-Reduction Potential

In order to titrate oxygen-senstive organic redox systems with reducing agents in solution (41), a special cuvette as shown in Figure 1 was designed. The flask used for preparation of the titrant is shown in Figure 2. These units were connected
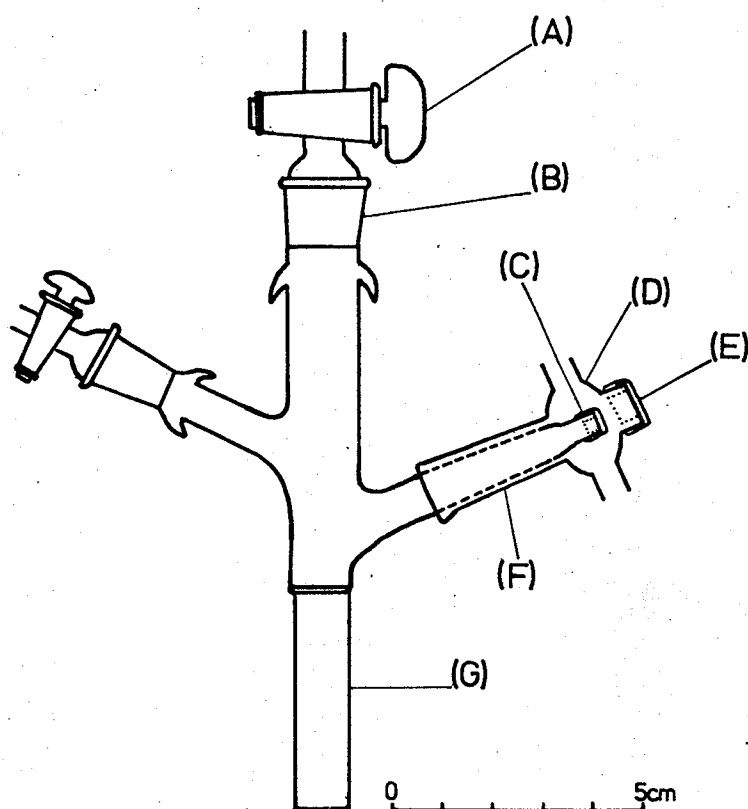
Figure 1. Cuvette unit: (A) High-vacuum stopcock, jointed to A-SGJ 14/15 standard taper male joint. (B) A-SGJ 14/15 standard taper female joint , fitted with a combined micro platinum electrode (EA,234, METROHM Ltd.).(C) A rubber gas chromatography septum. (D) The gas-lock fitting, through which oxygen free argon gas passes via an inlet and exit tube. (E) A rubber gas chromatography septum. (F) A female 10/24 standard taper joint. (G) The cuvette is made of glass.
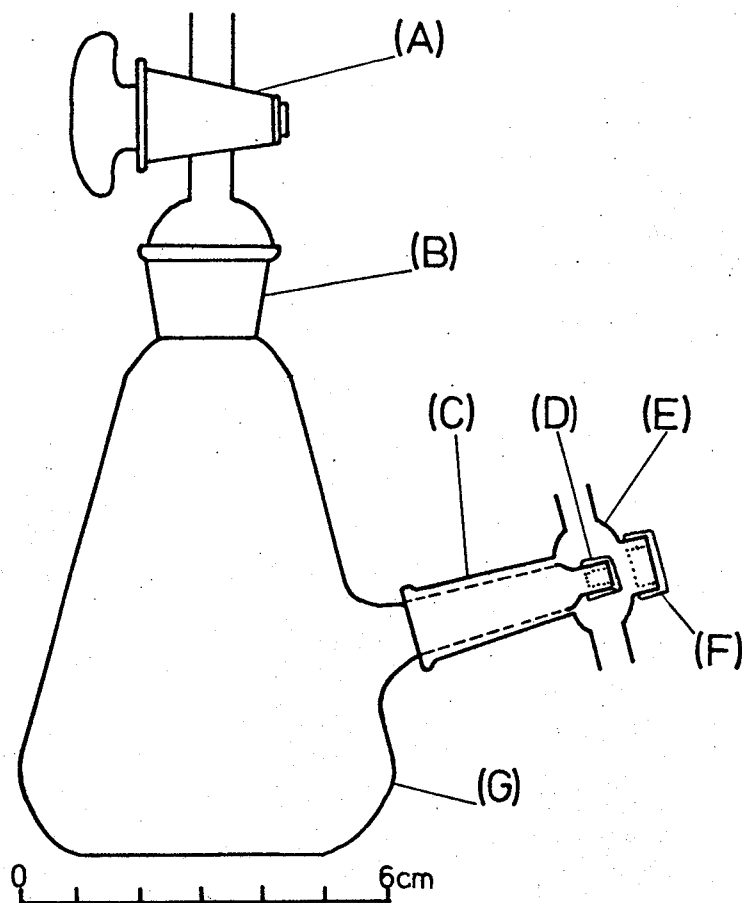
Figure 2. The flask: (A - F) are identical to those described for the cuvette unit in Figure 1.

to the argon gas line and vacuum line and arranged as shown in
Figure 3. After the redox system to be studied had been placed
in the cuvette and all free molecular oxygen purged from the
system by evacuation , a small amount of the titrating solution
which had been evacuated and flushed with an argon gas was deli-
vered from a Hamilton Gas-tight syringe through a rubber system.
To determine the redox potential a combined micro platinum
electrode (EA 234, Metrohm Ltd., Switzerland) and Hitachi-
Horiba pH meter were used. In the redox system, a catalytic
amount of various dyes was added as a mediator between the sys-
tem and the platinum electrode. All optical measurements were
carried out in a Cary recording spectrophotometer, model 16, or
a Union SM-401, high-sensitivity spectrophotometer, at $20^{\circ}C$.

Photoreduction of Chromatium cytochrome c-552 in the Presence
of EDTA

Chromatium cytochrome c-552 was placed in the main chamber
of Thunberg cuvette and made anaerobic by repeated cycles of
evacuation and flushing with an argon gas. Added flavin or
EDTA was maintained in the side arm during this process. After
the anaerobic conditions in the cuvette had been completed,
the solution in the side arm was mixed with that in the main
chamber before irradiation. The cuvette was then illuminated
with a 100 watt incandescent lamp at a distance of 10 cm from
the sample and at $25^{\circ}C$. Light was transmitted by water.

Experiments by the "Stopped-Flow" Method

Stopped-flow kinetic measurements of reduction of cyto-
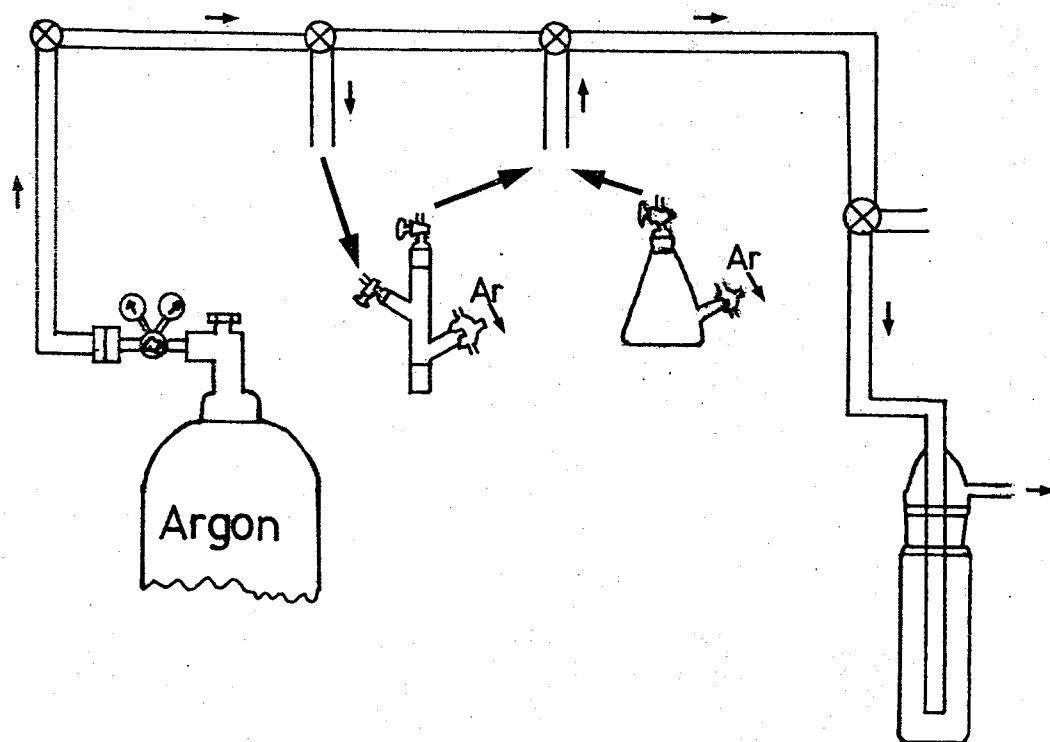chrome c-552 and horse cytochrome c by sulfide or dithionite

Figure 3. Connections to gas and vacuum lines. The method used to make the system anaerobic is as follows: The dithionite solution was prepared in the titrant flask (Figure 2). 50 ml of 20 mM K_2HPO_4, pH 10, was placed in the flask. After all free molecular oxygen had been purged from the solution with a vacuum pump, it was flushed with argon gas, stirring continuously with the magnetic stirrer. After two such purging cycles, the flask was opened and the required amount of dithionite (other reducing agent) was then added as the solid. The flask was quickly colsed and the solution was evacuated with the same method again. The solution to be titrated was prepared in the cuvette by the procedure like that used flask.

were performed in a Union stopped-flow rapid scan analyzer, model RA-601. Data analyses were made using a Union microcomputer system 77. The temperature was controlled at $25^{\circ}C$.

Measurements of Raman spectra

Raman spectra were obtained using the 514.5 nm line and 457.9 nm line of an argon ion laser (Spectra Physics, model 164) and a JEOL-400D Raman spectrometer equipped with an HTV-R649 photomultiplier. The frequency calibration of the Raman spectrometer was performed with indene (52).

RESULTS

Cultivation of Chromatium vinosum

A typical growth curve of C.vinosum is shown in Figure 4. At the log phase, a few globules of elemental sulfur accumulated were seen inside each cell (Figure 5). They disappeared at the stationary phase.

Oxidation of Sulfide with Chromatium cytochrome c-552

C.vinosum cytochrome c-552 purified in the present investigation by the method of Bartsch and Kamen (12,13) showed the absorption spectrum as shown in Figure 6. The absorption bands or shoulders at 450 and 475 nm in the oxidized form were attributable to the flavin. On reduction with $Na_2S_2O_4$, the absorption spectrum of usual cytochrome c was seen. The purified cytochrome c-552 was homogeneous as judged from the electrophoretic pattern; when it was subjected to polyacrylamide gel electrophoresis in the absence of SDS a single band was observed. Further, when the cytochrome preparation was subjected to iso-
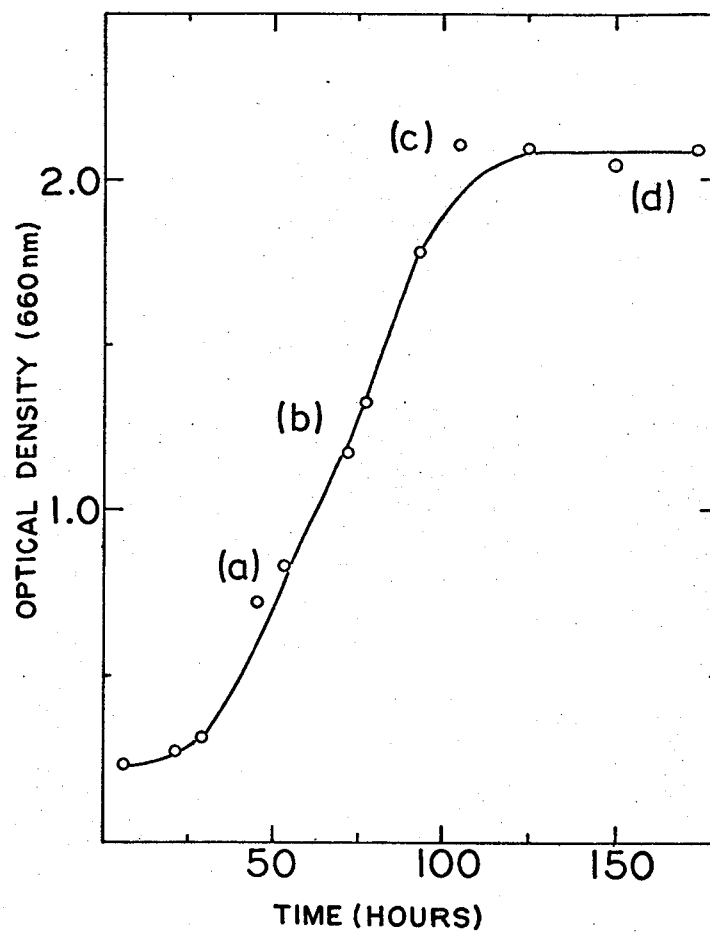
Figure 4. Growth curve of Chromatium vinosum. The bacterium was cultivated in the medium under the conditions as described in "MATERIALS AND METHODS". The turbidity was determined by the absorbance at 660 nm. Cells were harvested at the stationary phase after cultivation for 5 days.
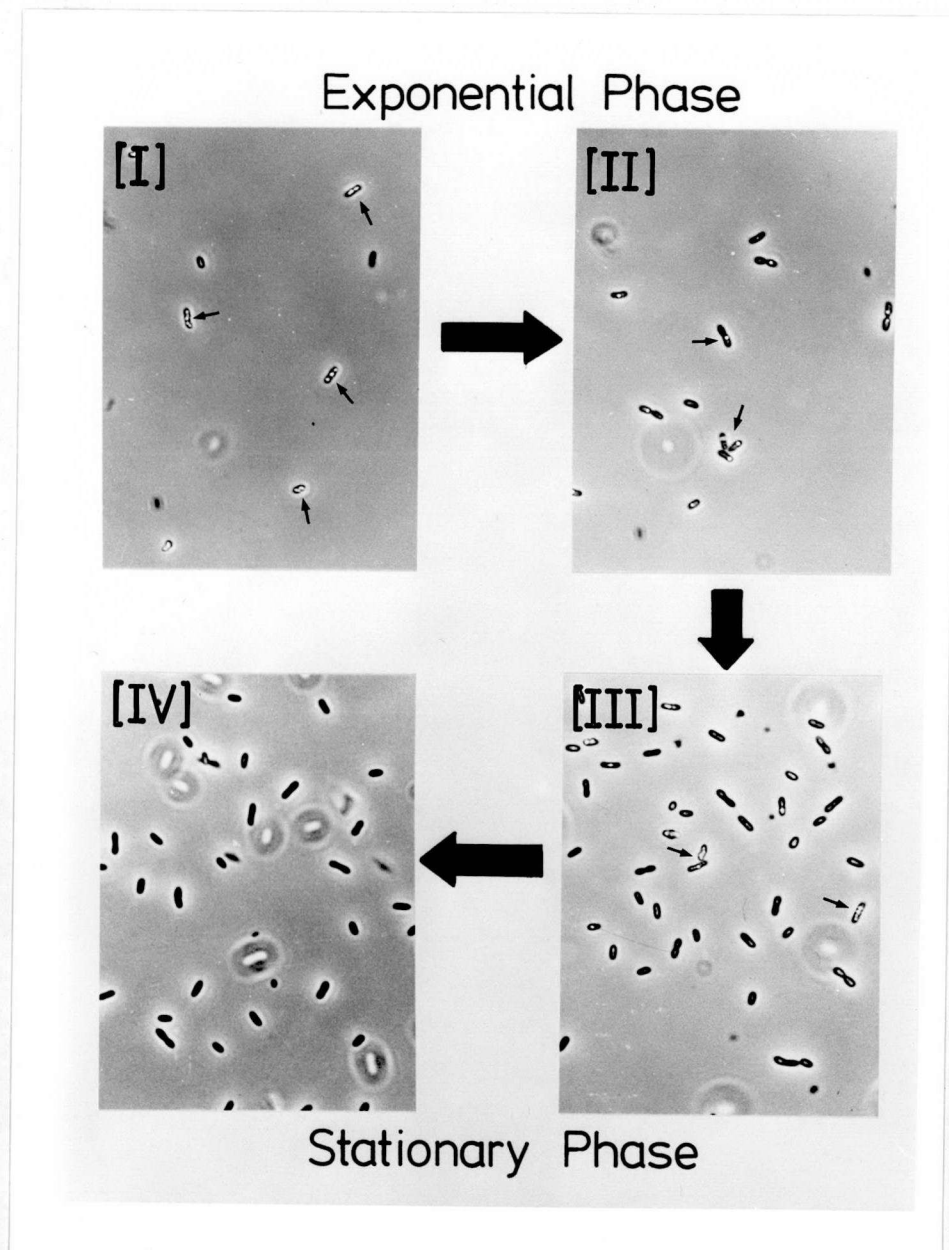
Figure 5. Pase-contrast photomicrographs of <u>Chromatium</u> <u>vinosum</u>
cells at various phases (all photographs, x 1,000). (I),Early-
exponential phase (Figure 4-a); (II),Late-exponential phase
(Figure 4-b); (III),Stationary phase (Figure 4-c); (IV),Stationary
phase (Figure 4-d). Cells contain the intracellular sulfur gra-
nules (I,II). With the growth of the bacterium, the granules
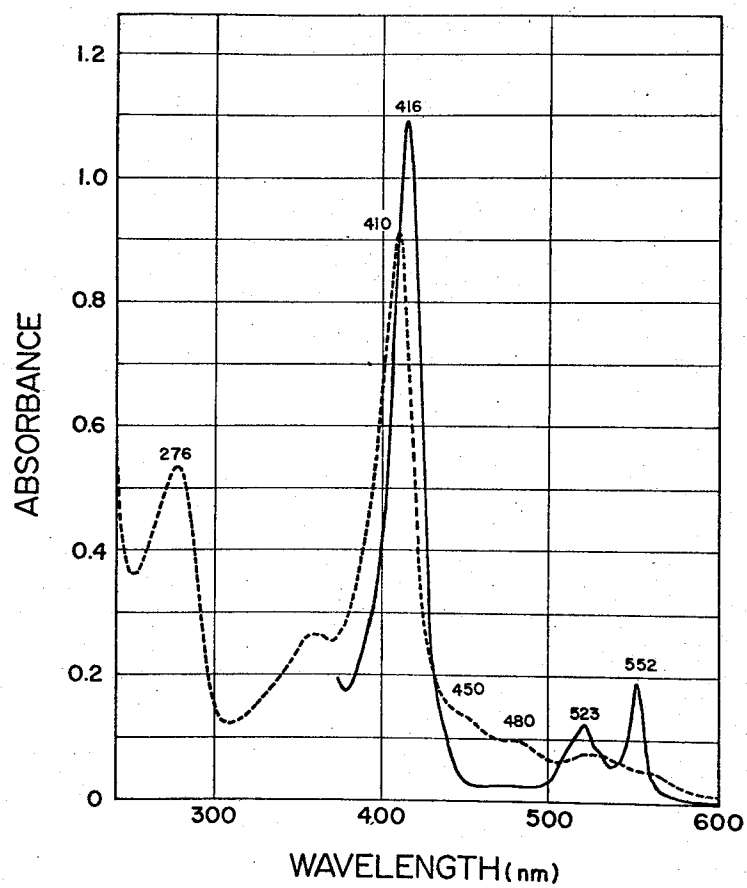disappear (III,IV).

Figure 6. Absorption spectra of the oxidized and reduced forms of *Chromatium* cytochrome c-552 (3.4 μM). The cytochrome was dissolved in 10 mM Tris-HCl buffer, pH 8.5.   -------, Oxidized; ————————,reduced with sodium dithionite.

electric focusing, only a single band was detected as determined from the absorbances at 280 nm and 410 nm of the eluate (Figure 7). This highly purified preparation of cytochrome c-552 showed a sulfide-cytochrome c reductase activity.

As shown in Figure 8, reduction of horse cytochrome c by Na_2S was greatly accelerated by the addition of a very small amount of cytochrome c-552. Cytochrome c was scarcely reduced by Na_2S at the concentration used in the present experiment unless cytochrome c-552 was added. The optimum pH of the reaction was 8.3 and Km of the flavocytochrome for sulfide was 12.5 μM. The reduction of cytochrome c with Na_2S in the presence of cytochrome c-552 was strongly inhibited by KCN. When the cytochrome was heated at $80^{\circ}C$ for 2 min, its catalytic activity greatly decreased. CO slightly affected the catalytic activity of the cytochrome. Some of the catalytic properties of cyto-chrome c-552 are summarized in Table I. Free FAD or FMN did not show the sulfide-cytochrome c reductase activity.

As shown in Table II, several c-type cytochromes were examined if they had the ability to act as electron acceptors in place of horse cytochrome c in the sulfide-cytochrome c reduction cata-lyzed by cytochrome c-552. Cytochrome c_2 (R.rubrum, Em,7 = 0.320 volt) and cytochrome c (552, N.europaea, Em,7 = 0.250 volt) acted as electron acceptors as efficiently as horse cyto-chrome c, while cytochrome c (554, P.aeruginosa, Em,7 = 0.225 volt) and cytochrome c (555, C.limicola f. thiosulfatophilum, Em,7 = 0.145 volt) were not reduced under the same experimental conditions. Cytochrome c' (Em,7 = 0.005 volt) and cytochrome
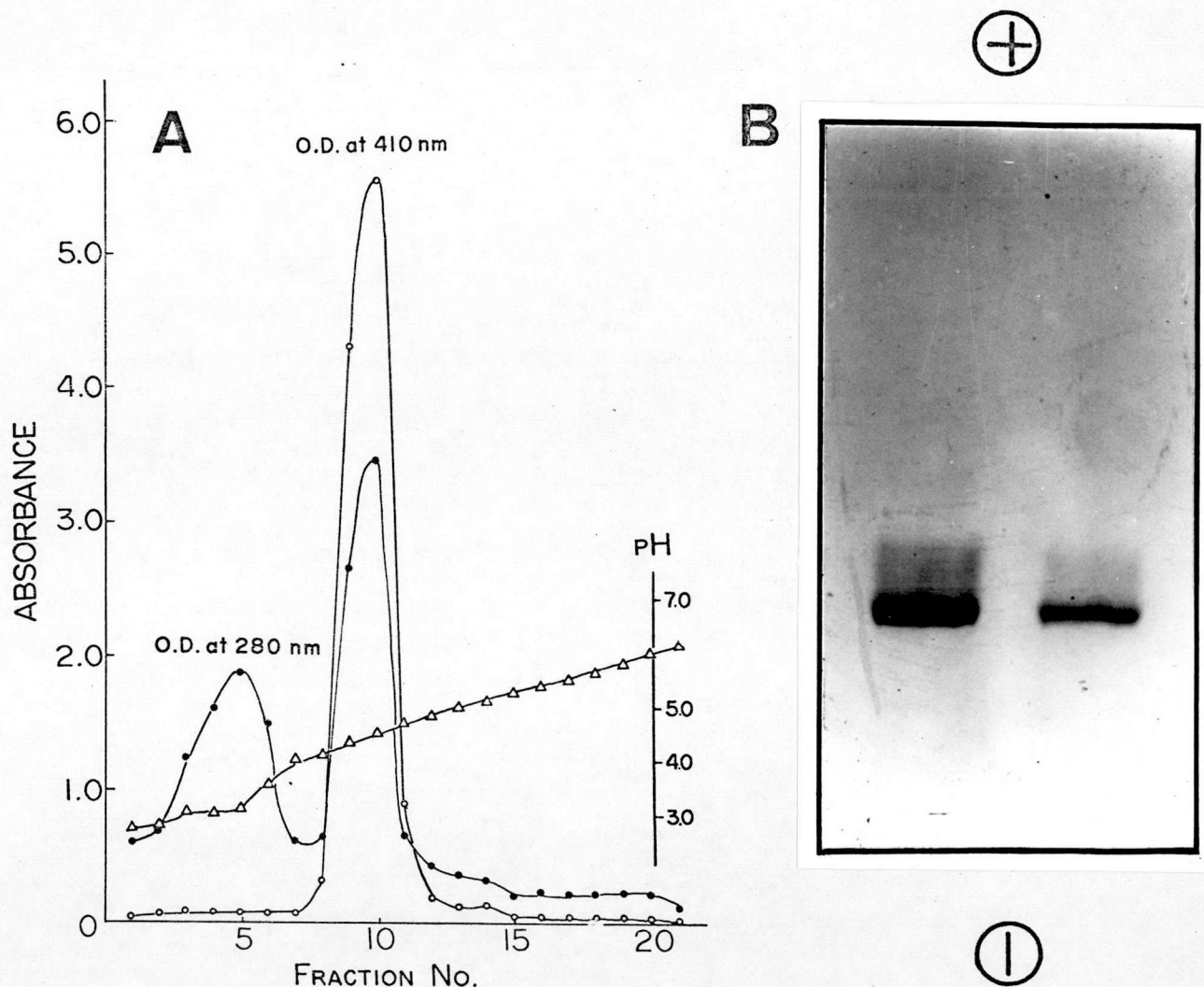
Figure 7. (A) Elution pattern of cytochrome c-552 after isoelectric fractionation. Isoelectric focusing was performed for 2 days with 1 % carrier ampholyte of pH range 3-10 using an apparatus with a volume of 110 ml. Although a peak was usually seen in the elution curve around pH 3.5 when the eluate was monitored in terms of the absorbance at 280 nm, it appeared to be attributable to materials derived from the carrier ampholyte used.
(B) Polyacrylamide gel electrophoretic patterns of the purified preparation of cytochrome c-552. The gel was prepared from 7.5 % acrylamide. The electrophoresis was performed in 0.1 M Tris-HCl buffer, pH 8.5, for 2 h at 20 mA and 150 volt, and at 4^{0}C. The two electrophoretic patterns were obtained from cytochrome c-552 at different concentrations. The cytochrome was stained with Coomassie brilliant blue.

-16-
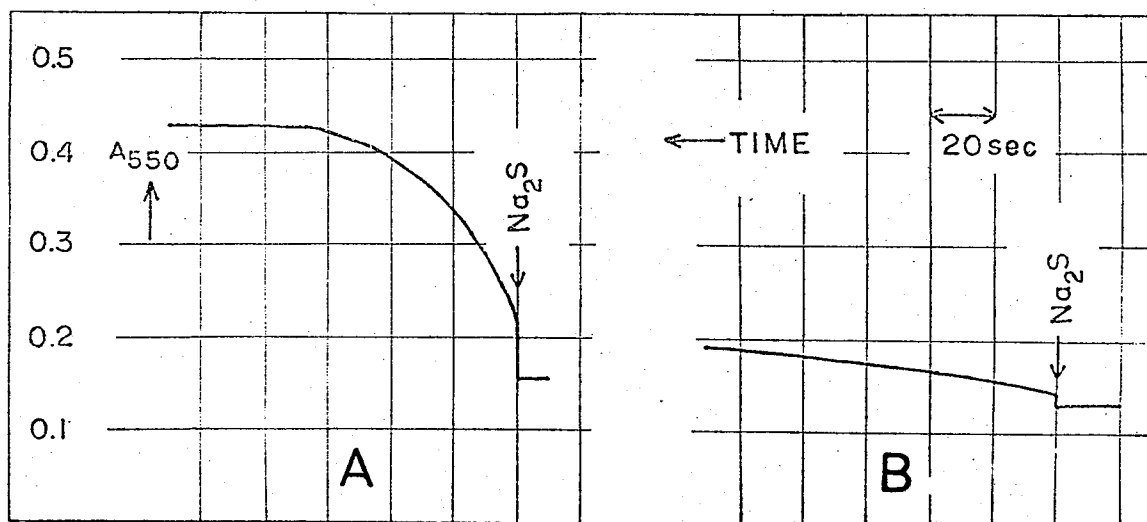
Figure 8. Time course of the reduction of horse cytochrome $\underline{c}$ by cytochrome $\underline{c}$-552 with Na_2S. The reaction mixture contained 0.2 M Tris-maleate buffer, pH 8.3, 19 μM horse ferricytochrome $\underline{c}$, 40 nM cytochrome $\underline{c}$-552, and 10 μM Na_2S, and performed in air at $23^\circ C$. A, complete reaction system; B, in the absence of cytochrome $\underline{c}$-552.

Table I. The sulfide-cytochrome c reductase activity of cytochrome c-552. The experimental conditions were as described in the legend for Figure 8, except for the concentration of Na_2S. The molecular activity was calculated from Vmax.

Conditions of the reaction	Molecular activity (mol of horse cytochrome c reduced/mol of cytochrome c-552/min)
Complete	7,140
+KCN 22 μM	4,210
670 μM	1,790
Cytochrome c-552 heated at 80°C, 2 min	1,790
Under CO atmosphere (1 atm.)	6,500

Table II. Reduction of several kinds of C-type cytochromes by cytochrome c-552 with Na_2S. The reaction mixture contained 10 mM Tris-HCl buffer, pH 8.5, 10 µM Na_2S, and each cytochrome with (+) or without (−) 20 nM C.vinosum cytochrome c-552 in a total volume of 1.0 ml. The reactions were started by adding Na_2S and performed at $20^\circ C$. The increase in the absorbance at the α−peak of each cytochrome was followed spectrophotometrically.

Cytochromes c		Addition of C.vinosum cytochrome c-552	Cytochrome reduced (nmol/min)
Source	α-Peak		
Horse	550	−	7.0
		+	37.2
Rhodospirillum rubrum	550	−	5.0
		+	24.8
Pseudomonas aeruginosa	554	−	5.7
		+	6.1
Chlorobium limicola f. thiosulfatophilum	555	−	1.6
		+	2.0
Nitrosomonas europaea	552	−	1.4
		+	12.0

c-553(550) (Em,7 = 0.330 volt) purified from C.vinosum did

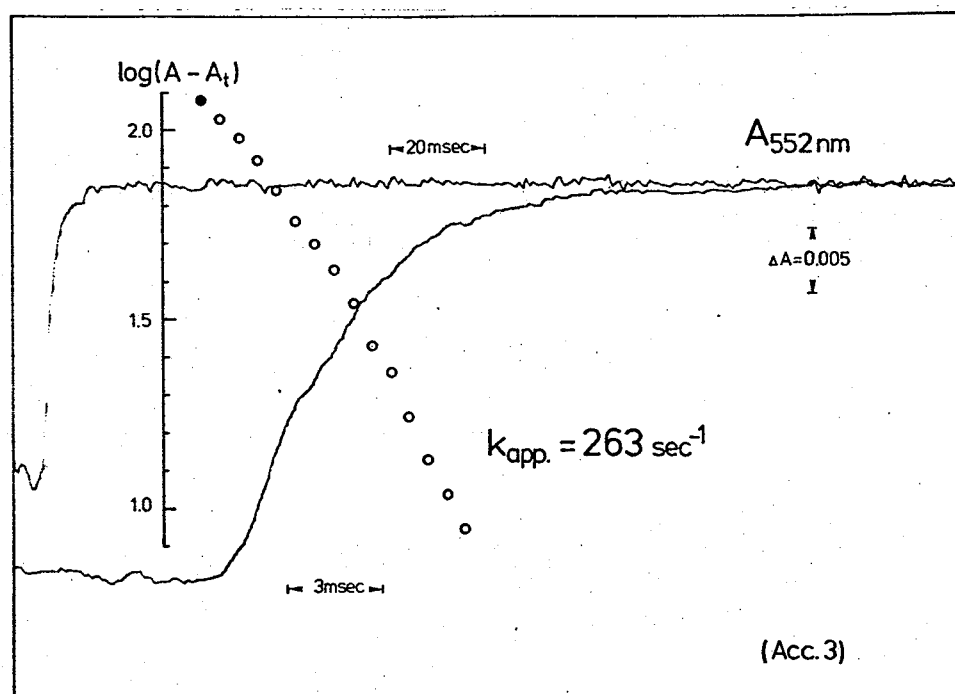not act as electron acceptors for cytochrome c-552, either.

Table III shows the stoichiometry in the reduction of horse

cytochrome c catalyzed by the cytochrome c-552 with sulfide.

The ratio of horse cytochrome c reduced to sulfide added was

2 : 1. In this experiment, horse cytochrome c was added in

excess of sulfide. These results suggest that sulfide is oxidi-

zed to elemental sulfur by the cytochrome c-552.

The reduction of Chromatium cytochrome c-552 and horse cyto-

chrome c with dithionite was followed with a stopped-flow appa-

ratus. The results are shown in Figure 9. The apparent first-

order rate constant for reduction of heme was obtained by plotting

$\log(A - A_t)$ against time. The rate constants in reduction of

cytochrome c-552 and horse cytochrome c were 263 sec^{-1} and 192

sec^{-1}, respectively. Reduction of cytochrome c-552 with sulfide

was also measured by stopped-flow (Figure 10). However, the

reduction was very fast so that the apparent first order rate cons-

tant could not be determined by the method as described above.

Therfore, the constant was calculated by use of $\underline{k} = \dfrac{0.693}{t_{1/2}}$,

where $t_{1/2}$ was half-value period. The constant, $\underline{k}$ thus obtained

was 530 sec^{-1}. The reduction rate of horse cytochrome c with

sulfide was determined and compared with that of cytochrome c-552.

The results are shown in Figure 11. As seen clearly from the

results shown in Figure 11, the reduction rate of cytochrome c-

552 with sulfide is much faster than that of horse cytochrome c.

However, the kinetic relationship in the reduction between flavin

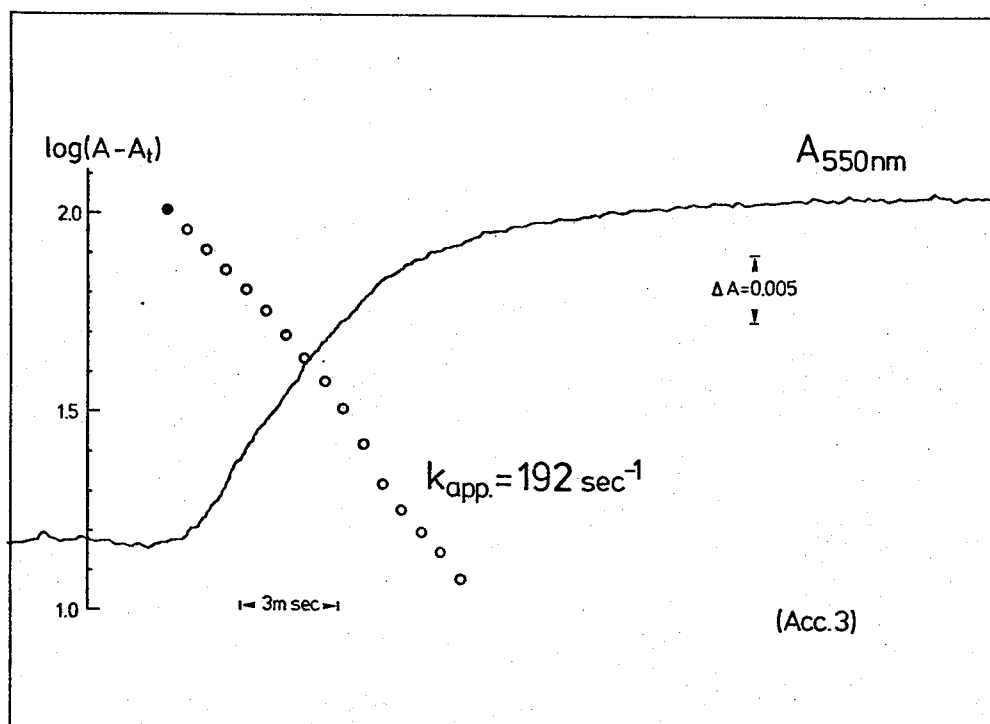and heme c of cytochrome c-552 could not be clarified because

Table III. Stoichiometry in reduction of horse cytochrome c catalyzed by Chromatium cytochrome c-552 in the presence of Na_2S. The reaction mixture contained 10 mM Tris-HCl buffer, pH 8.5, 9.1 µM horse cytochrome c, and 0.19 µM Chromatium cytochrome c-552 in a total volume of 3.1 ml.

Experiment	Na_2S added (nmole)	Horse cytochrome c reduced (nmole)
1	2.5	5.0
2	5.0	8.7
3	7.5	14.0
4	10.0	18.0
5	12.5	24.9

(A)

$\log(A - A_t)$

2.0

1.5

1.0

A_{552nm}

⊢20msec⊣

$\Delta A = 0.005$

$k_{app.} = 263 \text{ sec}^{-1}$

⊢ 3msec ⊣

(Acc. 3)

(B)

$\log(A - A_t)$

2.0

1.5

1.0

A_{550nm}

$\Delta A = 0.005$

$k_{app.} = 192 \text{ sec}^{-1}$
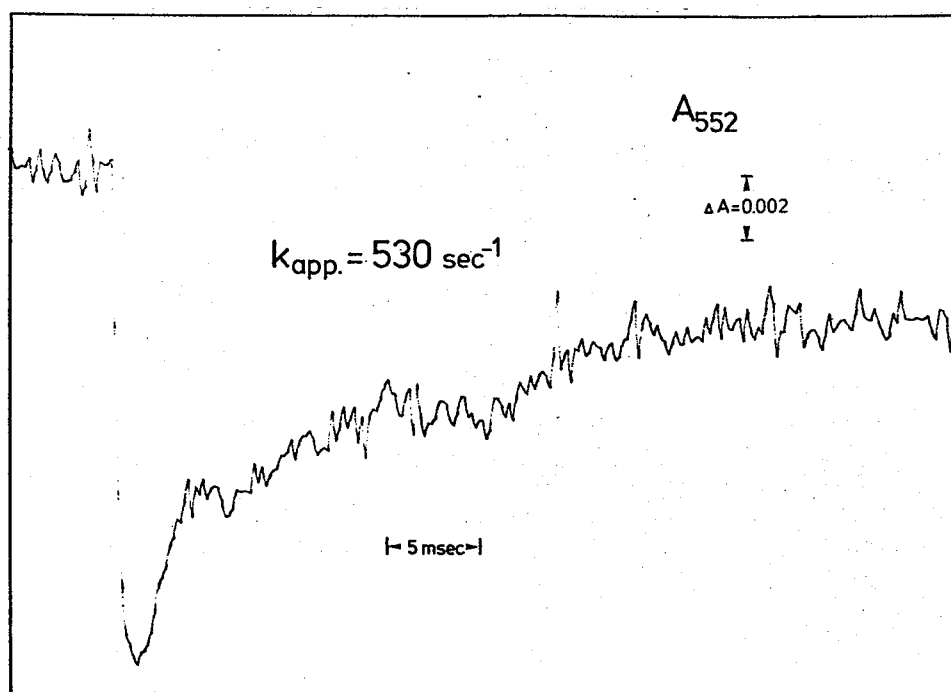
⊢ 3m sec ⊣

(Acc. 3)

Figure 10. Reduction of Chromatium cytochrome c-552 with sulfide, followed in a stopped-flow apparatus. The experimental conditions were as described in the legend for Figure 9, except that sufide in place of dithionite was added. The concentrations of Chromatium cytochrome c-552 and sulfide in the reaction mixture were 1.4 µM and 1 mM, respectively.
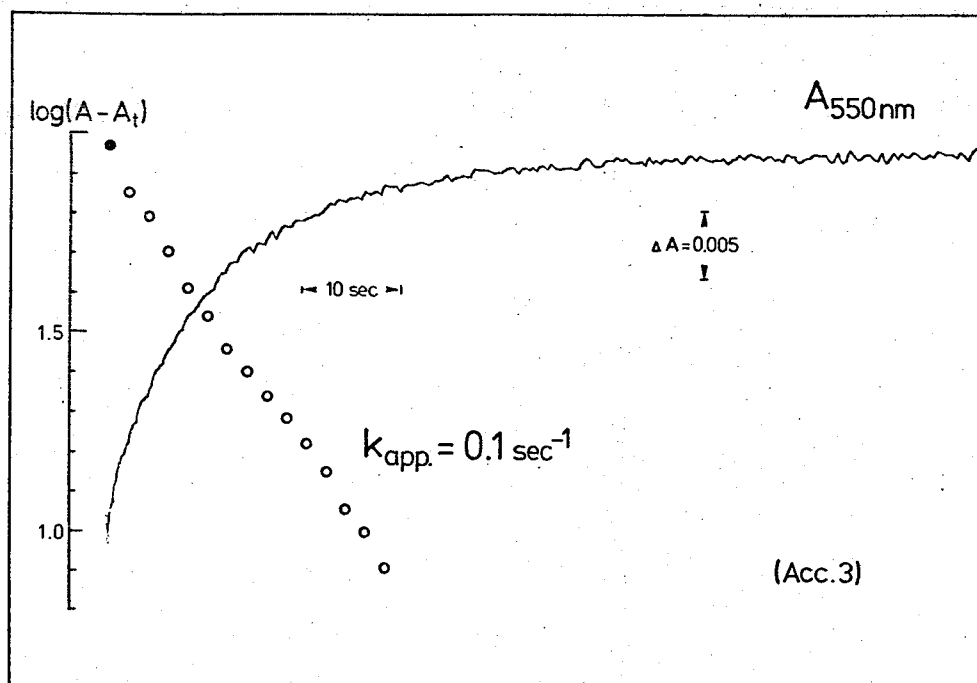
Figure 11. Reduction of horse cytochrome $\underline{c}$ with sulfide, followed in a stopped-flow apparatus. The experimental conditions were as described in the legend for Figure 9, except that sulfide was added in place of dithionite and that the concentrations of horse cytochrome $\underline{c}$ and sulfide were 2.7 μM and 1 mM, respectively.

of the very fast reduction of the cytochrome with sulfide and
of overlapping of the absorption spectrum of flavin with that
of heme.

Interaction of Sulfite and Cyanide with Cytochrome c-552

Massey et al. found (42) that an interesting reaction of
flavoproteins with sulfite. They showed that a number of flavo-
proteins were bleached on addition of sulfite and that the rea-
gent bound covalently and reversibly with N-5 of the flavin
nucleus (43). As Meyer et al. (53) have shown, the flavin of
cytochrome c-552 also reacts with sulfite and cyanide. In
the present investigation, their findings were confirmed by
measuring resonance Raman spectra. Figure 12 shows the resonance
Raman spectra of cytochrome c-552 in the presence of sulfite
and cyanide. Addition of sulfite or cyanide to cytochrome c-
552 resulted in change of Raman lines attributable to the flavin
of cytochrome c-552, while it did not affect Raman scattering
by the heme groups of the cytochrome. The characterization of
the Raman lines are summarized in reference (54,55).

Reduction of Elementary Sulfur with Chromatium cytochrome c-552

Cytochrome c-552 also catalyzed anaerobically reduction of
elementary sulfur with reduced benzylviologen as the electron
donor (Figure 13). The reaction was inhibited by cyanide.
The reaction mixture smelled of hydrogen sulfide and changed the
color of lead acetate test paper to brown after the reaction
had proceeded to some extent. Further, production of hydrogen
sulfide was confirmed by the methlene blue formation method
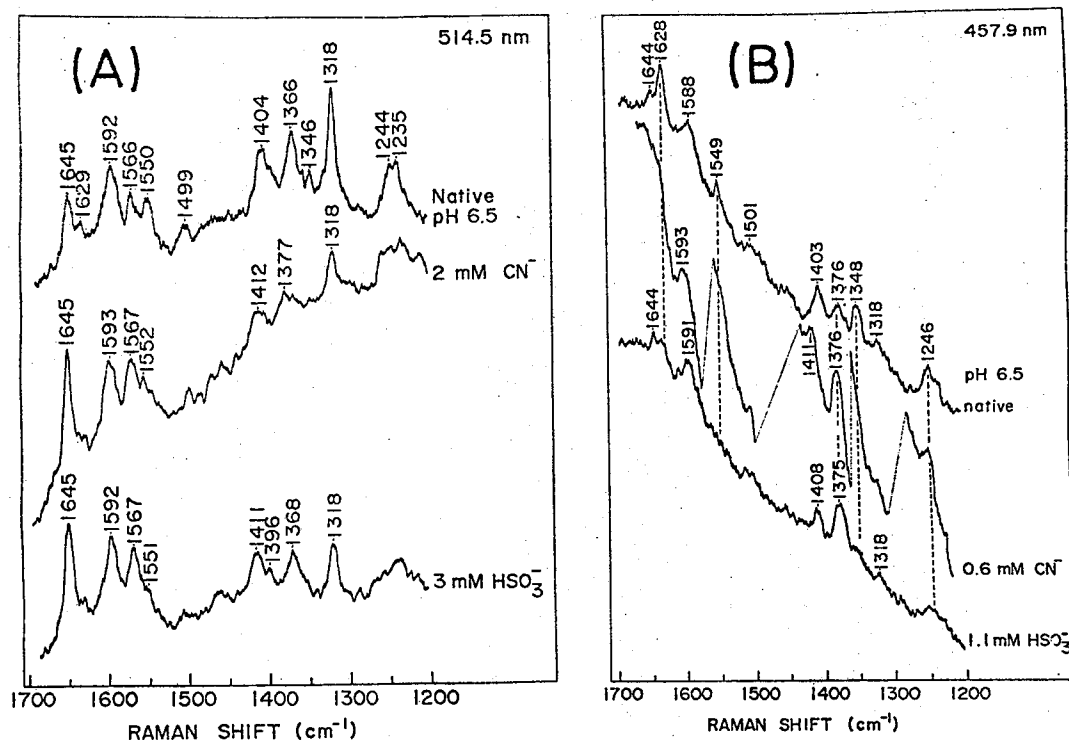with slight modifications as described in "MATERIALS AND METHODS"

Figure 12. Resonance Raman spectra of cytochrome c-552 in the presence of sulfite and cyanide. Reaction mixture contained 0.1 M sodium phosphate buffer, pH 6.5, 30 μM cytochrome c-552 and sulfite or cyanide at the concentrations indicated in the figure. A total volume of the reaction mixture was 60 μl. (A) When cytochrome c-552 was excited at 514.5 nm in the presence of sulfite and cyanide, Raman lines attributable to heme c did not change. (B) When excited at 457.9 nm under the same experimental conditions as (A), four Raman lines at 1628, 1549, 1348, and 1246 cm^{-1} disappeared. These lines are attributable to flavin molecule (55).
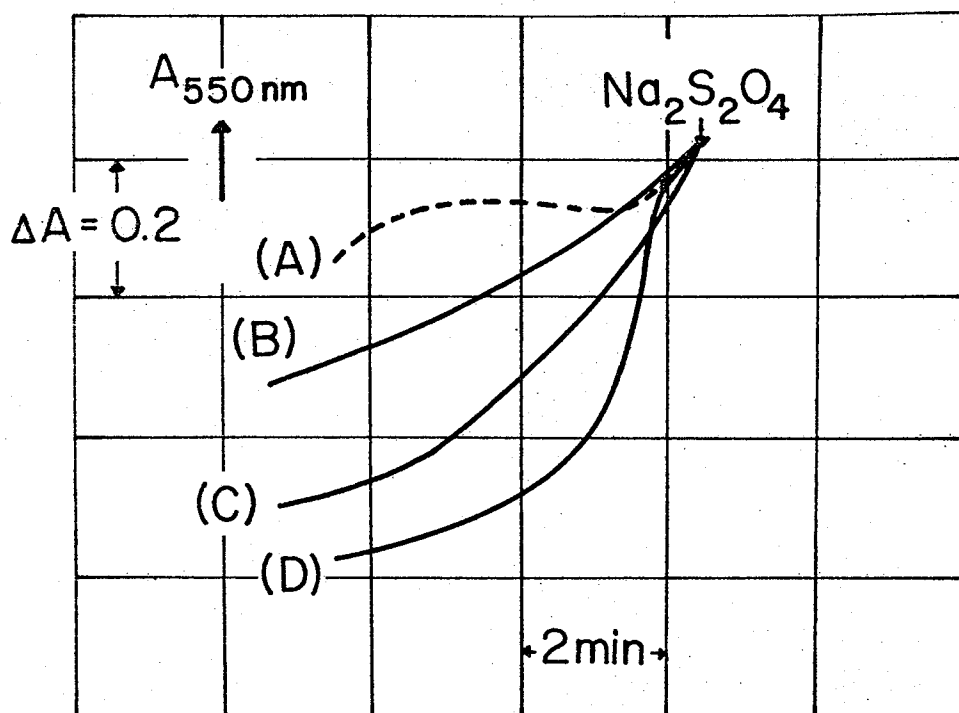
Figure 13. Anaerobic oxidation of reduced benzylviologen by
elementary sulfur catalyzed by cytochrome $\underline{c}$-552. The reactions
were preformed in Thunberg-type cuvettes. The reaction mixture
in the main chamber contained 10 mM Tris-HCl buffer, pH 8.5,
0.67 mM benzylviologen, and 0.94 μM cytochrome in a total volume
of 3.0 ml, and in the side chamber 1.2 μmol $Na_2S_2O_4$ was added as
a mixture with glucose ($Na_2S_2O_4$:glucose, 1:50). The reaction
was started by tipping the $Na_2S_2O_4$ into the reaction mixture in
the main chamber. Benzylviologen was reduced instantly when
$Na_2S_2O_4$ was mixed with the reaction mixture, and then the reduced
pigment was oxidized in the presence of elementary sulfur.
Although the purple color of reduced benzylviologen was unchanged
for more than 30 min in the absence of the cytochrome, the absor-
bance of the reaction mixture decreased gradually as elementary
sulfur precipitated readily. Elementary sulfur remained in the
suspension for more than several hours in the presence of cyto-
chrome $\underline{c}$-552 . The reference cuvette contained all the components
except for $Na_2S_2O_4$. When cyanide was added to the reaction mixture,
the reaction rates decreased.

(A), In the absence of cytochrome $\underline{c}$-552; (D), Complete reaction
system; (B) and (C), 12.2 and 6.1 mM KCN were added, respectively.

to avoid the inhibitory effect of dithionite on the analysis (44).
However, a stoichiometric relationship between consumption of
the reduced benzylviologen and sulfide formation has not yet
been established; as far as tested the molar ratio of reduced
benzylviologen consumed to sulfide formed was about 10, although
the ratio should theoretically be 2.

Photoreduction of Chromatium cytochrome c-552

Free flavin or some of the enzyme-bound flavin are photo-
reduced under anaerobic conditions in the presence of adequate
electron donors e.g. EDTA, while C-type cytochromes are not
reduced under same conditions. Chromatium cytochrome c-552
was slightly photoreduced in the presence of EDTA as shown in
Figure 14, while addition of free FAD or FMN stimulated greatly
the photoreduction as shown in Figure 15. The absorption spec-
trum of photoreduced cytochrome c-552 was the same as that of
the dithionite reduced cytochrome. Figure 16 illustrates the
resonance Raman spectra of the cytochrome c-552 preparations;
these preparations involve the cytochrome as purified and
the cytochrome dialyzed, reduced with dithionite, and irradiated
with or without EDTA before the determinations of the spectra.
When the oxidized cytochrome c-552 was anaerobically exposed to
laser illumination (60 milliwatt, 35 min) in the absence of EDTA,
its Raman spectrum did not change. When the resting enzyme was
anaerobically subjected to laser illumination (60 mW, 35 min)
in the presence of EDTA, the Raman lines at 1347 cm^{-1} and 1246
cm^{-1}, which were attributable to oxidized flavin, disappeared,
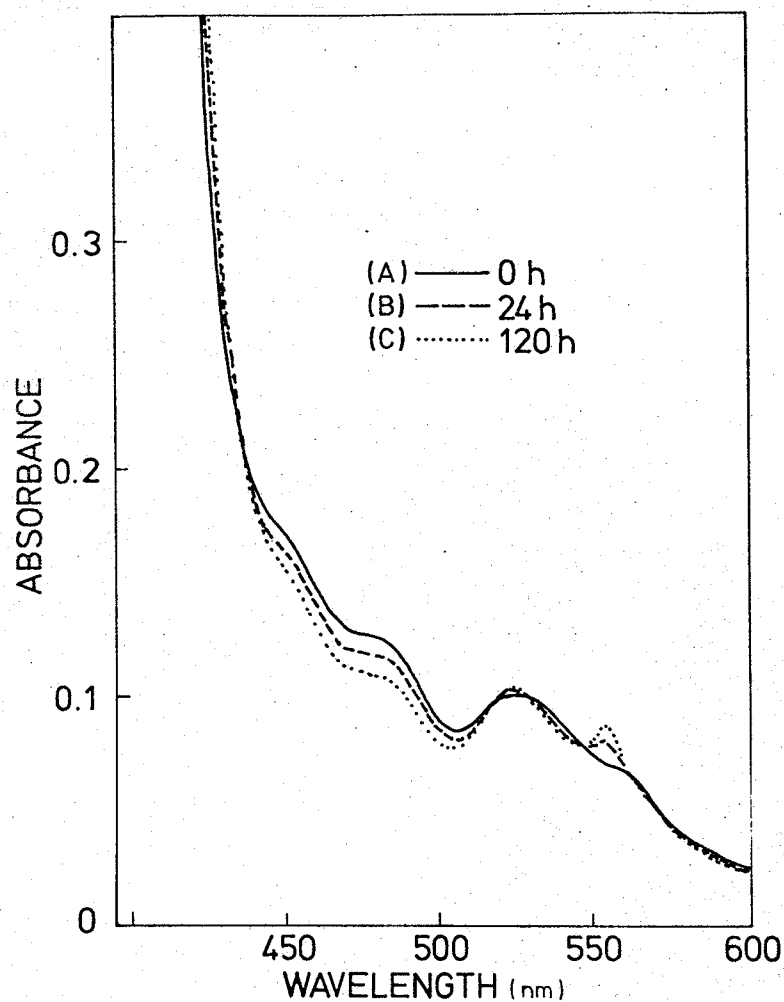while the Raman line at 1364 cm^{-1}, which was attributable to

Figure 14. Photoreduction of Chromatium cytochrome c-552.
The reactions were performed in Thunberg-type cuvettes. The main
chamber contained 3.9 µM cytochrome c-552 dissolved in 3.2 ml
of 0.1 M sodium-phosphate buffer, pH 7.0, and the side chamber
0.15 ml of 0.1 M EDTA. Experimental conditions were as described
in "MATERIALS AND METHODS". (A), before illumination; (B), after
24 h illumination; (C) after 120 h illumination.
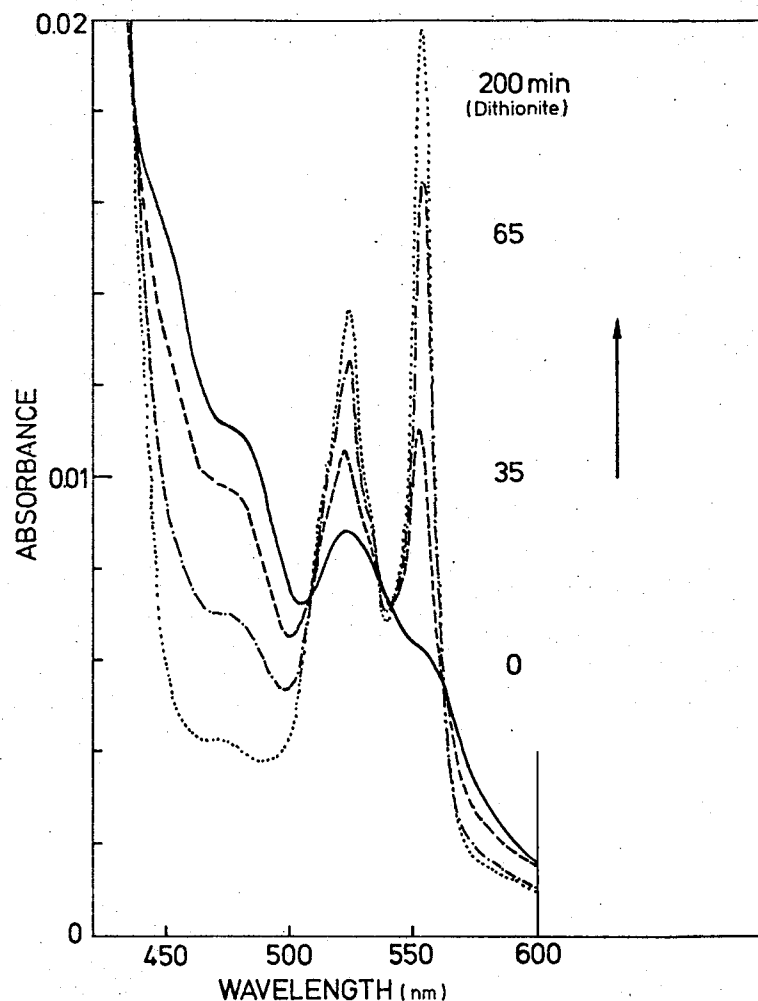
Figure 15. Photoreduction of Chromatium cytochrome c-552 in
the presence of FAD. The reactions were performed in Thunberg-
type cuvettes. The main chamber contained 3.7 μM cytochrome c-
552 and 0.29 μM FAD dissolved in 3.2 ml of 0.1 M sodium-phosphate
buffer, pH 7.0. The side chamber contained 0.1 ml of 0.1 M EDTA.
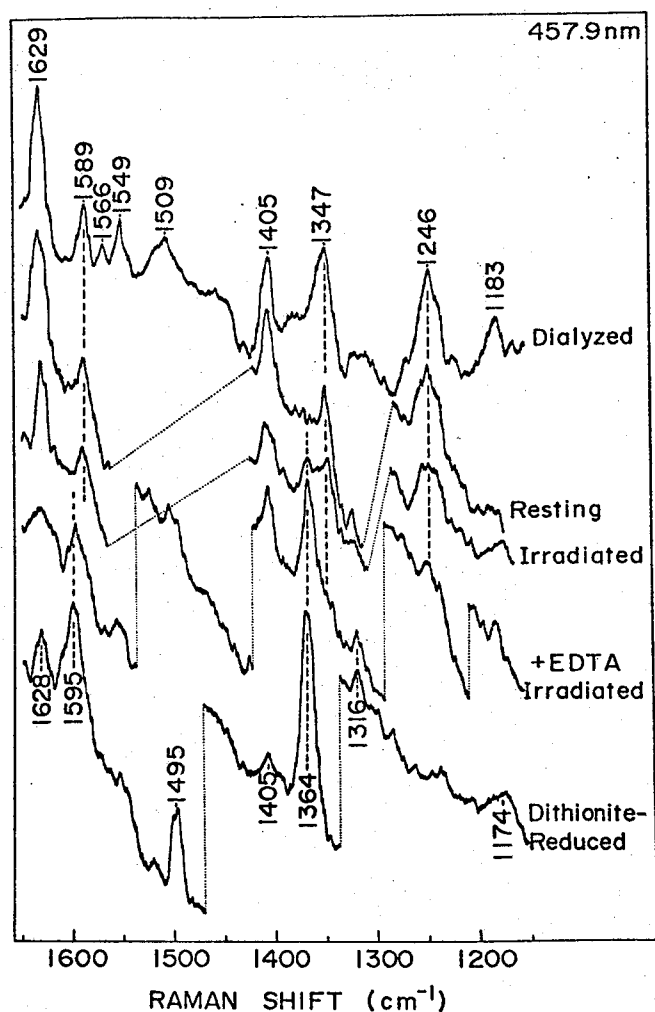Experimental conditions were as described in "MATERIALS AND METHODS".

Figure 16. Resonance Raman spectra of oxidized, reduced and irradiated preparations of cytochrome c-552. "Dialyzed" means the cytochrome which was dialyzed against 0.1 M Tris-HCl buffer, pH 8.5 for 12 h. "Resting" means the cytochrome as purified. "Irradiated" means the cytochrome which was irradiated with laser in the absence of EDTA. "Irraidated + EDTA" means the cytochrome which was irradiated with laser in the presence of EDTA. The reaction mixture contained 0.1 M Tris-HCl buffer, pH 8.5, 70 μM cytochrome c-552, and 5 mM EDTA in a total volume of 60 μl. These cytochrome preparations were excited at 457.9 nm.

Laser powers are common to five spectra; 60 mW for irradiation (0.5 h) and 20mW for measurements.

reduced heme c, appeared. The resonance Raman spectrum of cyto-
chrome c-552 irradiated in the presence of EDTA was the same as
that of cytochrome c-552 reduced with dithionite. As flavoprotein
is reduced by irradiation in the presence of EDTA but cytochrome
c is not, these results suggest that electron is transferred
from flavin to heme c in the molecule of cytochrome c-552.

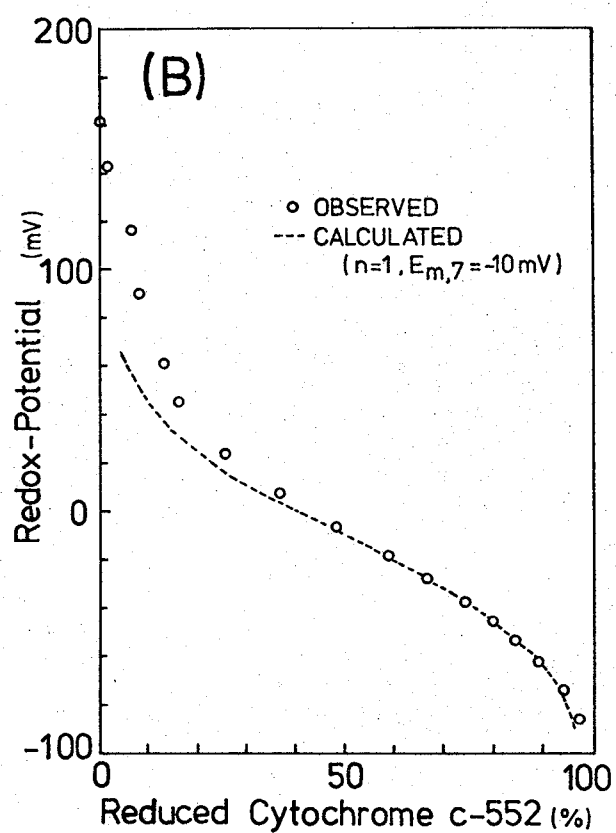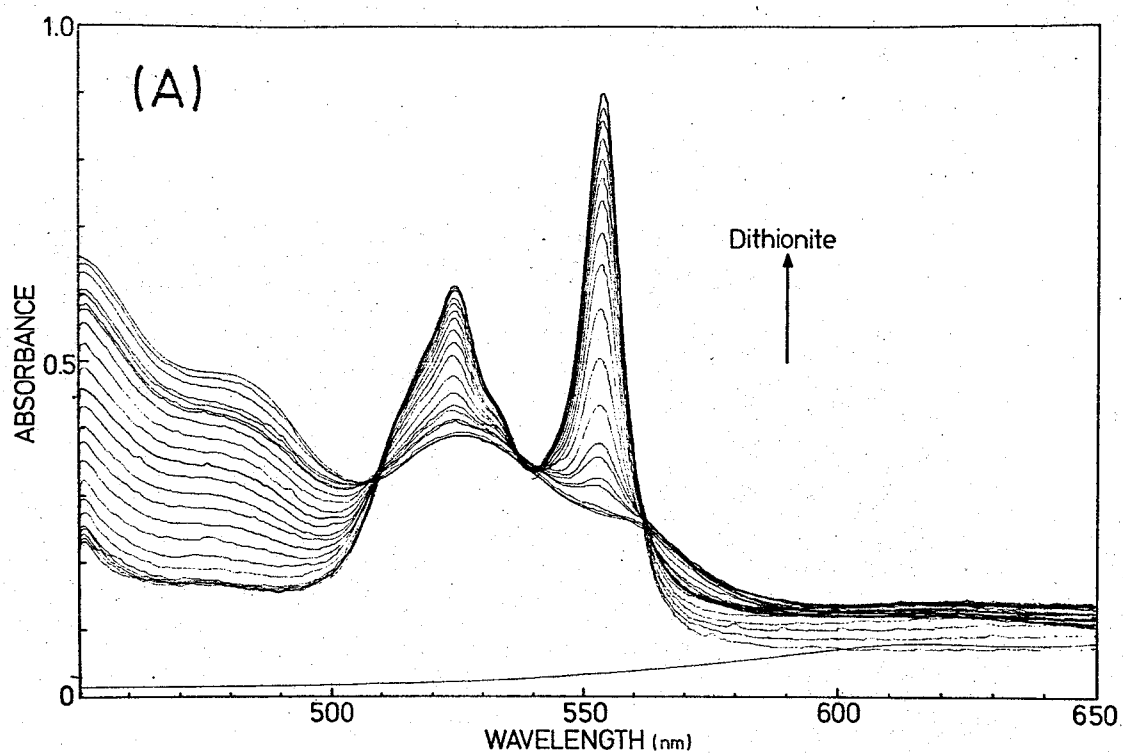Oxidation-Reduction Potential of Cytochrome c-552

The oxidation-reduction potential of cytochrome c-552 was
measured by the method as described in "MATERIALS AND METHODS".
First, the midpoint potential of phenosafranin ($E_m,7 = -0.252$
volt) was determined by dithionite titration to standardize the
electrode in a spectropotentiometrical cell as shown in Figure 1.
Secondly, the midpoint potential at pH 7 of horse cytochrome c
was determined by using the standardized electrode. In determi-
nation of the potential of horse cytochrome c, a catalytic amount
of DCIP was added in the redox system at the concentration where
its absorption spectrum of neither the oxidized nor reduced form
interfered with the spectrum of horse cytochrome c. Figure 17(A)
shows the change of the absorption spectra of horse cytochrome c
during titration with dithionite. The $E_m,7$ of horse cytochrome
c was determined to be 0.252 volt (Figure 17(B)). This value was
in good agreement with that reported by other workers. The poten-
tial of Chromatium cytochrome c-552 was determined by the same method
as in the case of horse cytochrome c except that DCIP and MV
were added as mediators in the redox system. The results obtained
with cytochrome c-552 are shown in Figure 18(A). The $E_m,7$ value
of cytochrome c-552 was determined to be -0.01 volt and "n" was

(A)

ABSORBANCE

Dithionite

WAVELENGTH (nm)

(B)

OXIDATION-REDUCTION POTENTIAL (mV)

○ OBSERVED
----- CALCULATED
(n=1, $E_{m,7}$ = 252 mV)

REDUCED CYTOCHROME C (%)
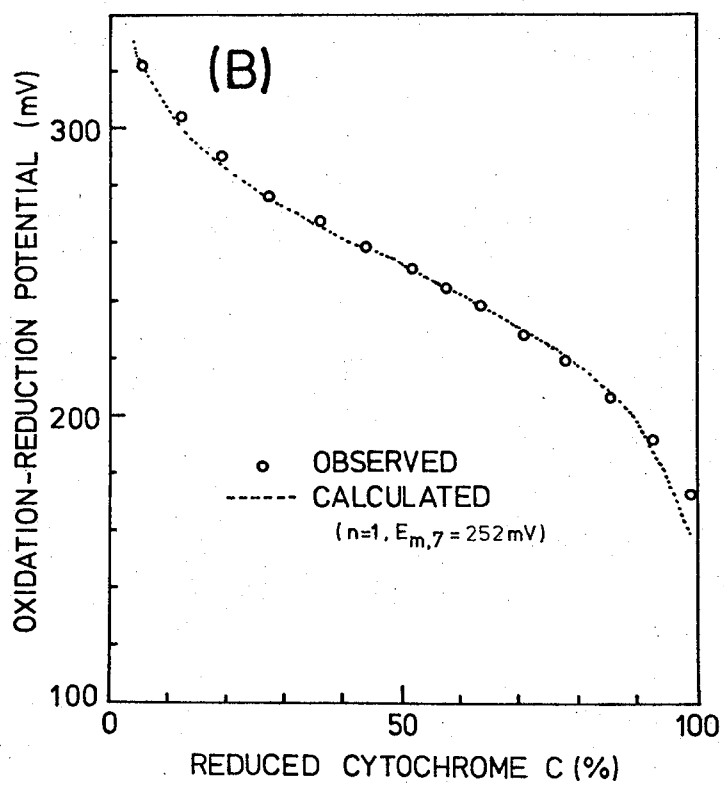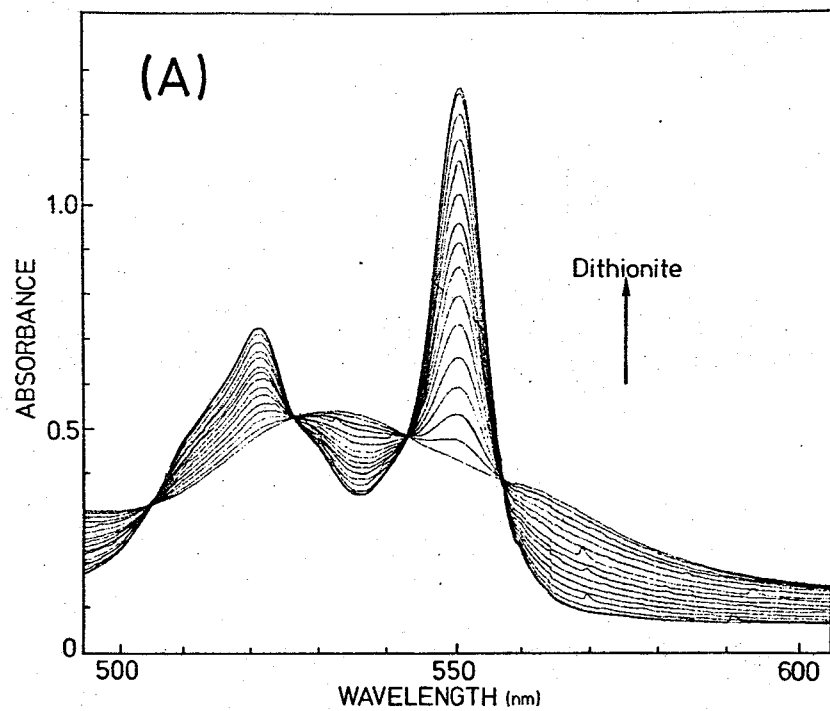
unity (Figure 18(B)). However, the Em,7 value obtained above
is the apparent one because cytochrome c-552 contains two mole-
cules of heme c in one molecule. Therefore, the midpoint potentials
of the two heme molecules in the cytochrome c-552 molecule were
determined. It is necessary to assume that the potentials of two
hemes obey independently the Nernst equation and the extinction
coefficients at 552 nm of two hemes are the same values both in
the fully reduced and fully oxidized forms. On such assumptions,
the relationship between the oxidation-reduction potential of
the system and the absorbance at 552 nm of the cytochrome may be
written as follows:

$$
\frac{1}{1 + e^{\frac{F(E-E_H)}{RT}}} + \frac{1}{1 + e^{\frac{F(E-E_L)}{RT}}} = \frac{2(A - A_0)}{A_\infty - A_0} \quad ,
$$

where, E, oxidation-reduction potential of the system (measured
value); E_H, E_L, midpoint potentials of each heme c molecule in
the cytochrome c-552 molecule; F, Faraday's constant = 23062.4
cal/volt; R, gas constant = 1.987 cal/deg; T, measured tempera-
ture = 293 degree; A, absorbance at 552 nm; A_0, absorbance at
552 nm of fully oxidized cytochrome c-552; A_∞, absorbance at 552 nm
of fully reduced cytochrome c-552. The values of E_H and E_L
satisfied this equation were obtained by substituting the mea-
sured values for the other variables; E_H and E_L obtained were 16.1mV
and -32.7 mV, respectively. A theoretical titration curve obtained
using these values is shown in Figure 19. The theoretical curve
almost coincided with the experimental one. This confirms that

(A)

Dithionite

WAVELENGTH (nm)

ABSORBANCE

(B)

o OBSERVED
--- CALCULATED
   (n=1 , $E_{m,7}$ = -10 mV)

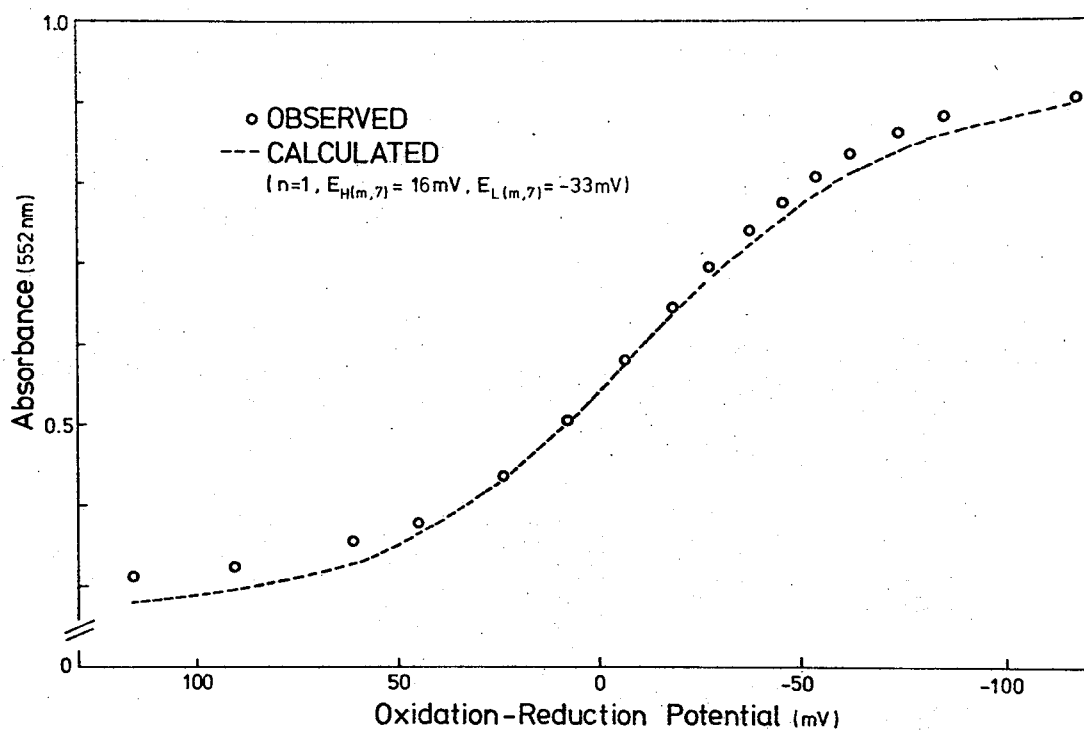Redox-Potential (mV)

Reduced Cytochrome c-552 (%)

Figure 19. Theoretical absorbance changes of cytochrome c-552 in reduction with sodium dithionite. The theoretical titration curve was obtained assuming that midpoint potentials of two hemes of cytochrome c-552 were 16 mV and -33 mV, respectively and that "n" was one. (o), observed points, (---), theoretical curve.

the midpoint potentials of the two hemes are about 16 mV and

-33 mV, respectively, on the assumptions as described above.

On the oter hand, the midpoint potential of flavin of cytochrome

c-552 could not be determined by the same method as in case of

heme, as the percentage of the flavin in the reduced form could

not be estimated from the absorbance change at an appropriate

wavelength in the absorption spectrum. Singer et al. reported

that the midpoint potential of flavin — peptide derived from

cytochrome c-552 was -187 mV (47).

Reductive Titration of Chromatium cytochrome c-552 with Sulfide

The reductive titration of cytochrome c-552 was performed

with its substrate, sodium sulfide. Figure 20 illustrates the

change of the absorption spectra of cytochrome c-552 during

titration with sodium sulfide. Figure 21 shows the absorbance

changes at 552 nm and 480 nm in the spectrum of the cytochrome

during reduction with sulfide. The changes in terms of percent-

age of total absorbance were plotted against the amount of sul-

fide added. The lag in the titration curve was attributed to

traces of oxygen remained in the system. The absorbance at 552

nm was mainly attributable to heme, while 30 % of the absorbance

at 480 nm to flavin. The contribution in the absorbance at 480

nm by the flavin was calculated on the basis of the extinction

coefficient at the wavelength of free FAD. It was difficult to

determine spectrophotometrically the respective oxidation-reduc-

tion states of the two kinds of prosthetic groups in cytochrome

c-552, because of overlap of the absorption spectra attributable

to the flavin and heme. Although there were such difficulties,
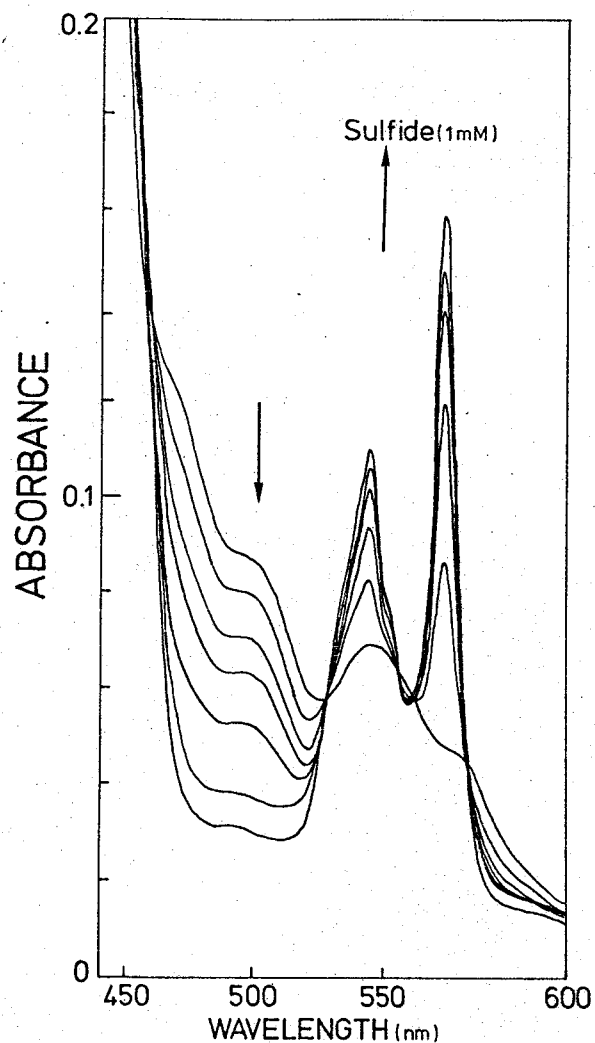
Figure 20. Anaerobic reductive titration of Chromatium cytochrome
c-552 with sulfide. Sulfide (1.0 mM) as titrant was dissolved
in 0.1 N NaOH. The reaction mixture contained 0.1 M Tris-HCl
buffer, pH 8.5, and 5.7 µM cytochrome c-552 in a total volume of
3.2 ml. The titration was performed by the method as described
in "MATERIALS AND METHODS".

Figure 21. Absorbance changes at 552 nm and 480 nm observed during reductive titration of cytochrome c-552 (5.3 µM) with sulfide (0.6 mM). Absorbance is plotted in terms of percentage of the total absorbance change. Experimental conditions were as described in "MATERIALS AND METHODS".

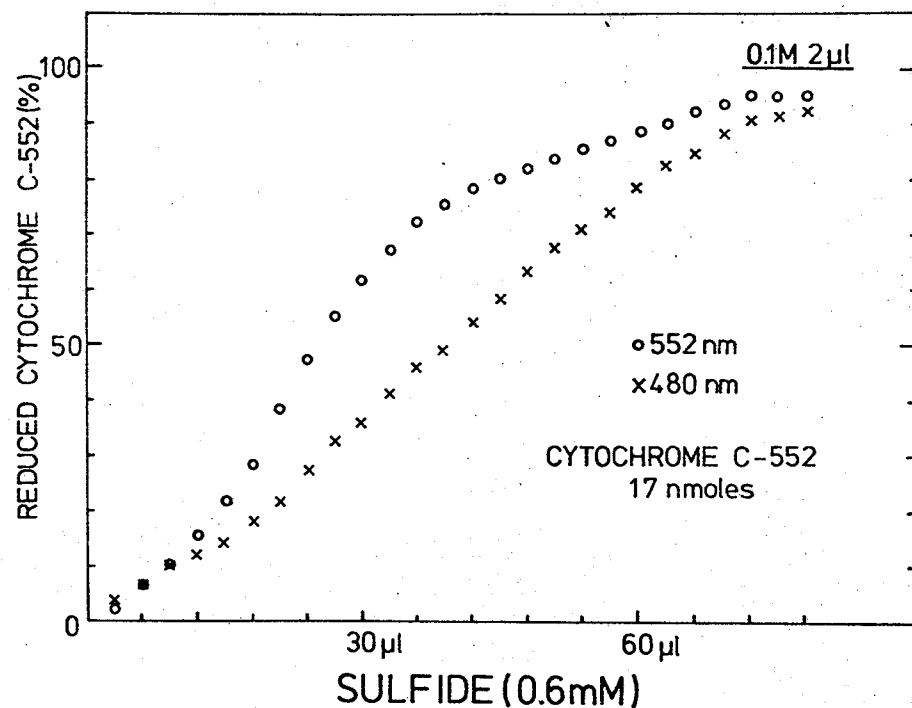the present experimental results suggested the possibility that the reduction percentage of the heme was always higher than that of the flavin. This is expectable from the midpoint potentials of heme c and flavin. If the midpoint potential of the flavin was higher than that of the two hemes, the absorbance change reverse to that shown in Figure 21 should be obtained.

Molecular Features

Cytochrome c-552 possesses one molecule of FAD and two molecules of heme c per molecule (19-21). Splitting the cytochrome c-552 molecule into subunits was tried to elucidate the relationship between its catalytic activity and structure. When cytochrome c-552 was subjected to polyacrylamide gel electrophoresis in the presence of 0.5 % SDS, 6M urea, and 1 % β-mercaptoethanol, two major bands and two minor bands were observed in gel stained with Coomassie brilliant blue R-250 (Figure 22). The molecular weights of the two major bands were 46,000 and 21,000, respectively, (Figure 23). The proteins included in the two bands with molecular weights of 46,000 and 21,000 were identified as the flavoprotein and cytochrome moieties, respectively, by separate electrophoresis of the preparations of these two moieties obtained as described below. The other two minor bands were attributed to contaminants or irregularity in the staining. As described below, this was supported by analyses of the N-terminal amino acid and the amino acid compositions of the intact cytochrome c-552 and two moieties.

When the cytochrome was subjected to isoelectric focusing for 2 days in the presence of 6M urea and 1 % β-mercaptoethanol
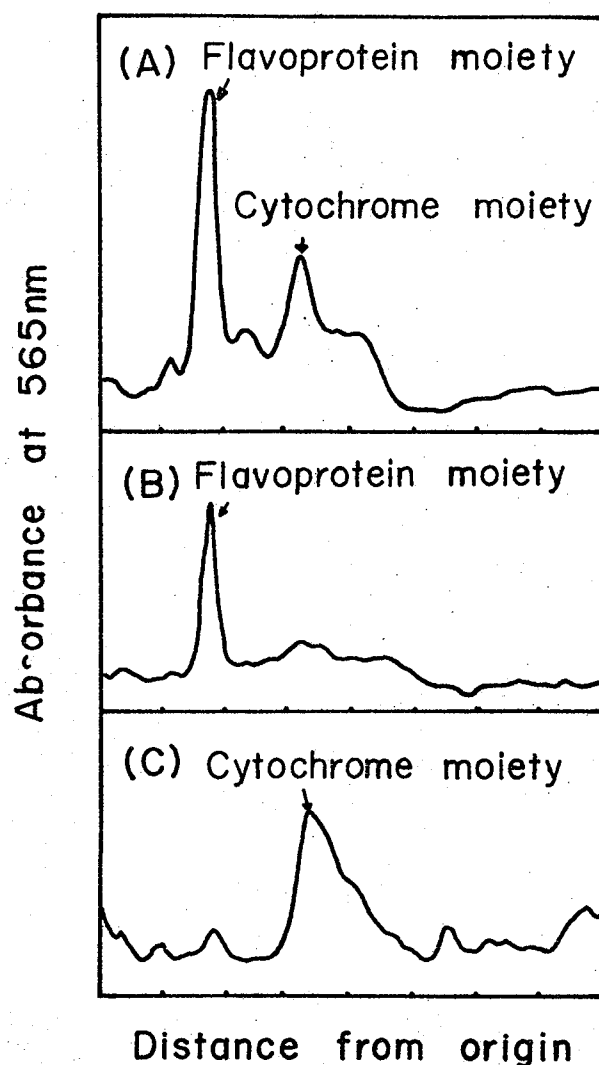
Figure 22. Electrophoretic profiles of cytochrome c-552 and
its subunits. Electrophoresis was performed in the presence of
0.5 % SDS, 6 M urea, and 1 % β-mercaptoethanol. When the intact
cytochrome c-552 was subjected to electrophoresis, two major and
two minor bands were observed in the gel. The molecular species
in the two major bands were identified as the cytochrome and flavo-
protein moieties, respectively, by separate electrophoresis of these
moieties (B and C). The two minor bands were attributed to some
contaminants or irregularity in the staining (see the text).
(A),cytochrome c-552; (B), the flavoprotein moiety; (C), the cyto-
chrome moiety.
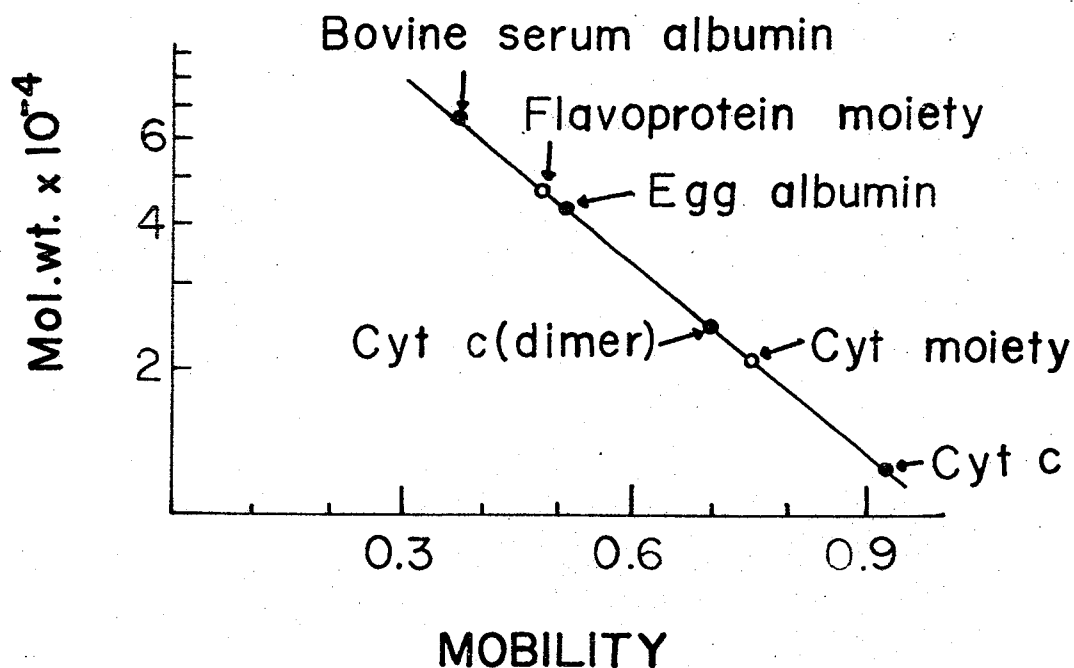
Figure 23. Molecular weight estimations of the cytochrome and flavoprotein moieties derived from cytochrome c-552 by polyacrylamide gel electrophoresis in the presence of 0.5 % SDS, 6 M urea, and 1 % β-mercaptoethanol. Their molecular weights were found to be 46,000 and 21,000, respectively. The proteins used as markers to determine the molecular weights of these moieties: horse cytochrome c (mol.wt. 12,300), egg albumin (mol.wt. 43,000), and bovine serum albumin (mol.wt. 68,000).

in addition to sucrose and carrier ampholyte, two peaks were found in the elution curve as monitored in terms of the absorbance at 280 nm (Figure 24). One peak was located at pH 5.3 and the other at pH 5.6. The eluate with pH 5.6 was yellow in color and showed absorption peaks at 276, 335, and 453 nm in the oxidized form (Figure 25). On reduction with dithionite, the absorption peak at 453 nm disappeared, while no peak was observed around 410 nm. These spectral properties show that the yellow eluate contains flavoprotein but not hemoprotein. The ratio of $A_{276 \ nm}/A_{453 \ nm}$ was 8.9 with this fraction. The yield of the flavoprotein moiety was more than 95 % on the basis of the absorbance at 450 nm. The flavoprotein moiety was not reduced with sulfide. The cytochrome moiety could not be obtained by the isoelectric focusing as mentioned above. In the presence of β-mercaptoethanol, the heme of the cytochrome moiety was very quickly destroyed, so that no red band was seen as would be expected if the cytochrome moiety was present in the electrophoretic column.

The cytochrome moiety was obtained by gel filtration with Sephacryl S-200 in 0.1M Tris-HCl buffer, pH 8.5, containing 6M urea and 0.1M KCl. The elution pattern is shown in Figure 26. The cytochrome c-552 preparation was dialyzed against 0.1M Tris-HCl buffer, pH 8.5, containing 6M urea and 0.1M KCl, for 12 h before being subjected to gel filtration. As shown in Figure 26, essentially two peaks were found in the elution curve as monitored in terms of the absorbance at 280 nm. The first and second peaks of the elution curve contained the flavoprotein moiety plus unsplit flavocytochrome c and the cytochrome moiety, respectively,
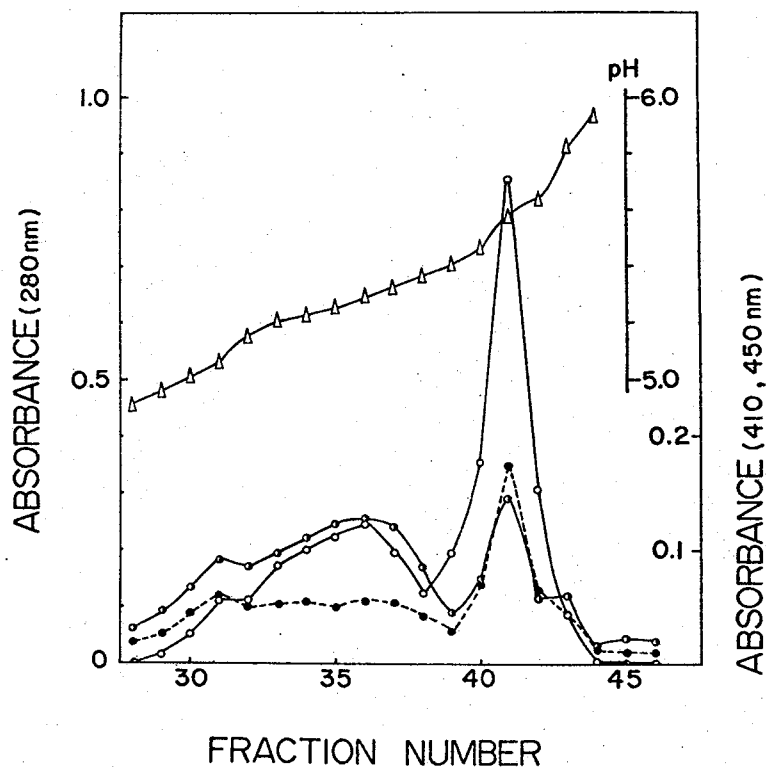
Figure 24. Isoelectric focusing of cytochrome c-552 in the pre-
sence of 6 M urea and 1 % β-mercaptoethanol. The electrophoresis
was performed with 1 % carrier ampholyte of pH range 3-6 for 16 h
at 1.7 mA and 800 volt using the apparatus with a volume of 110 ml.
The amount of cytochrome c-552 applied was 71 nmol. Each fraction
of the eluate was 2.0 ml. The absorbance was measured at 280 nm
(o), 450 nm(•), and 410 nm(o).

Figure 25. Absorption spectrum of the flavoprotein moiety derived from cytochrome $\underline{c}$-552. The moiety was dissolved in 10 mM Tris-HCl buffer, pH 8.0. (A), Oxidized; (B), reduced with $Na_2S_2O_4$.
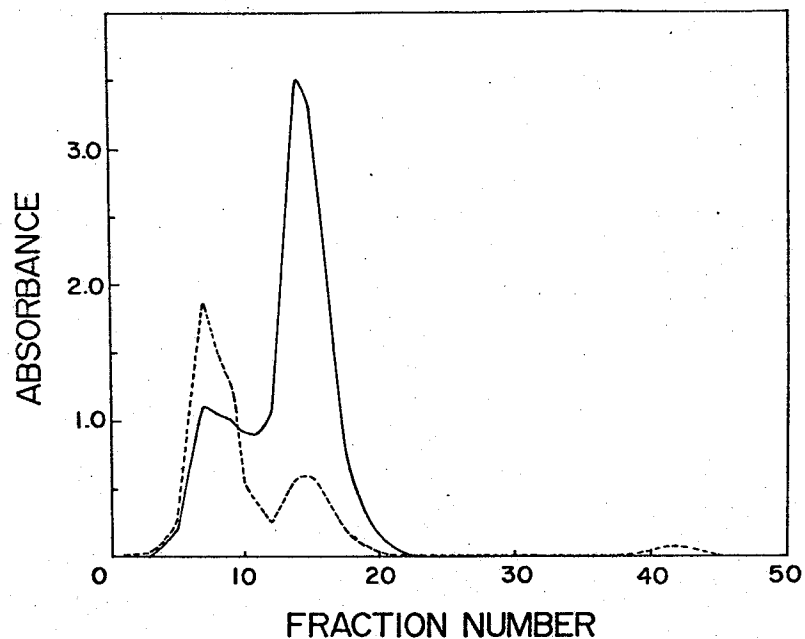
Figure 26. Elution pattern of the cytochrome moiety derived from cytochrome c-552. Cytochrome c-552 which had been dialyzed against 0.1 M Tris-HCl buffer, pH 8.5, containing 0.1 M KCl and 6 M urea was chromatographed on a Sephacryl S-200 column (2 x 62 cm) equilibrated with the same buffer used for the dialysis.
--------, 280 nm; ————, 410 nm.

as judged from their absorption spectra and the electrograms
obtained on polyacrylamide gel electrophoresis in the presence
of SDS. In this procedure, the cytochrome moiety was obtained
in a pure state, while the flavoprotein moiety was eluted to-
gether with the intact cytochrome which survived the procedure.
Figure 27 shows the absorption spectrum of the cytochrome moiety.
There were absorption peaks at 552, 523, and 416 nm in its reduced
form. The pyridine ferrohemochrome of the moiety showed absorp-
tion peaks at 550, 520, and 414 nm. The absorbance of the peak
around 280 nm of the cytochrome moiety was considerably low as
compared with that of the peak of the intact flavocytochrome;
the ratio of $A_{275 \, nm}$(oxidized)/A_{Soret}(oxidized) was 0.18 with
the cytochrome moiety, while the ratio with the intact cytochrome
was 0.56. The yield of the cytochrome moiety was about 70 % on
the basis of the absorbance at 552 nm.

The existence of the two subunits in the Chromatium cyto-
chrome c-552 molecule was confirmed by analysis of the N-terminal
amino acid residues. The first step of the Edman degradation
procedure gave alanine and glutamic acid and the second step
proline and glycine with the intact flavocytochrome. N-terminal
amino acid analysis of the flavoprotein moiety gave alanine followed
by glycine, while that of the cytochrome moiety gave glutamic
acid followed by proline. In the analyses of the N-terminal
residues, amino acids other than those described were practically
indectable.

The amino acid compositions of cytochrome c-552 and of the
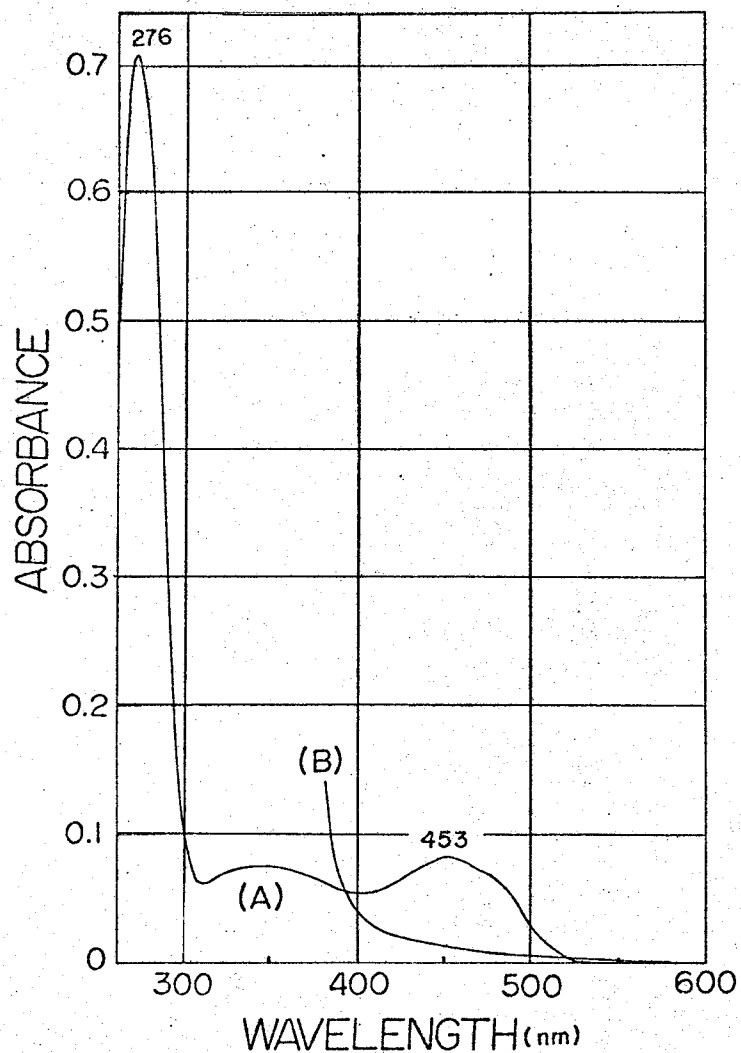cytochrome and flavoprotein moieties derived from it are shown
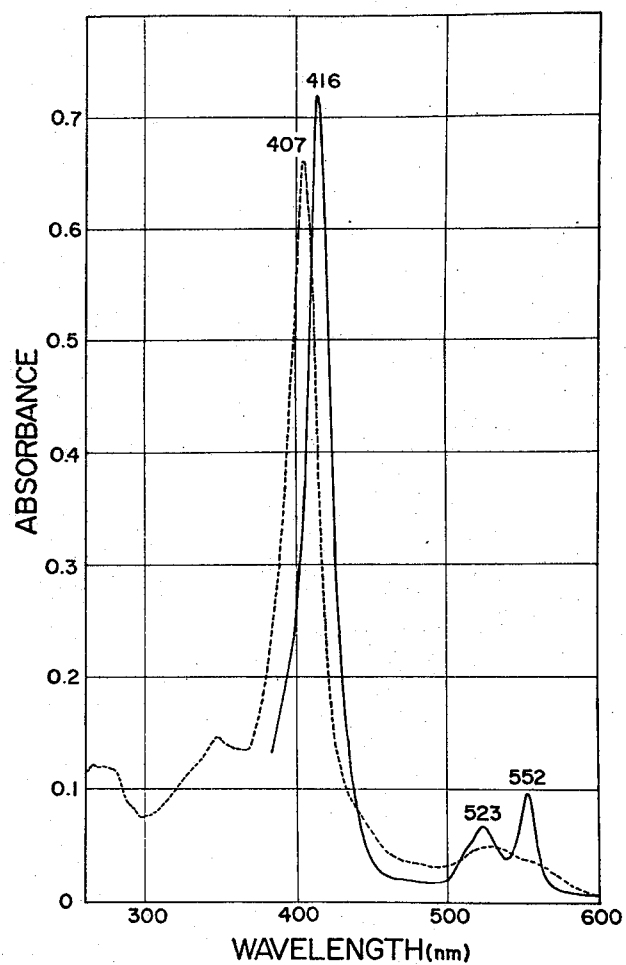
-47-

Figure 27. Absorption spectrum of the cytochrome moiety derived
from cytochrome c-552. The moiety was dissolved in 0.1 M Tris-HCl
buffer, pH 8.5, containing 6 M urea.
------, Oxidized; ————, reduced with $Na_2S_2O_4$.

in Table IV. The amino acid compositions shown in Table IV were determined on the basis of the molecular weights of the respective proteins. The molecular weights of the intact cytochrome c-552 and of the two moieties were determined on the basis of the heme content and by SDS-gel electrophoresis, respectively. The compositions calculated on the basis of mol of heme and FAD present in the preparations gave molecular weights of 62,000 and 34,000 for the native cytochrome c-552 and the flavoprotein moiety, respectively. The FAD content was determined assuming the millimolar extinction coefficient at 453 nm of the flavoprotein moiety to be 11, and the heme content was determined using a coefficient at 550 nm of the pyridine hemochrome of heme c of 29.1. The molecular weight thus obtained of the flavoprotein moiety differs considerably from that determined by SDS-gel electrophoresis. This discrepancy suggests that the assumed value of the extinction coefficient of the flavoprotein moiety is incorrect. The molecular weight of the hemoprotein moiety was determined to be 13,000 on the basis of the heme content, while it was determined to be 21,000 by SDS-gel electrophoresis. Therfore, it was concluded that the hemoprotein moiety as obtained by the above method contained two molecules of heme c per molecule. This is in good agreement with the results obtained by Bartsch et al. that Chromatium cytochrome c-552 has one molecule of FAD and two molecules of heme c per molecule, although they reported that the molecular weight is 72,000 ± 6,000. The molecular weight of cytochrome c-552 was determined to be 53,000 by gel filtration with Sephadex G-100 in the present study. In any case, the amino acid compo-

Table IV. Amino acid compositions of cytochrome c-552 and of the cytochrome and flavoprotein moieties. Numbers of amino acid residues were calculated on the basis of the molecular weights of each component determined as described in the text, and are expressed as the nearest integers. Tryptophan residues were not estimated.

Amino acid residue	Cytochrome c-552	*Cytochrome c-552	Flavoprotein moiety	Cytochrome moiety
Lys	39	42	29	13
His	14	16	10	5
Arg	16	18	13	8
Asp	48	52	38	15
Thr	25	26	19	10
Ser	32	33	27	11
Glu	58	63	39	24
Pro	37	39	27	9
Gly	56	63	46	17
Ala	67	74	51	22
Cys	7	8	6	3
Val	38	40	33	6
Met	14	15	5	7
Ile	31	36	27	8
Leu	31	35	23	12
Tyr	26	29	20	6
Phe	17	19	11	8
Total	556	608	424	184
**mol.wt.	61,800	67,400	44,700	21,800

*Calculated on the basis of the molecular weight obtained by summation of the molecular weights of the two moieties. ** Values including heme and/or flavin.

sition of the intact cytochrome c-552 was in good agreement with the sum of these of the two moieties.

After the two subunits obtained above had been separately dialyzed against 10 mM Tris-HCl buffer, pH 8.5, their sulfide-cytochrome c reductase activity was measured. Neither subunit showed the activity. Further, the activity was not restored even when a mixture of the two subunits was dialyzed against 10 mM Tris-HCl buffer, pH 8.5.

Photoreduction of the cytochrome subunit and flavin protein subunit was studied. By a preliminary experiment, the cytochrome subunit was not photoreduced but the flavin protein subunit seemed to be photoreduced in the presence of EDTA.

DISCUSSION

Chromatium cytochdrome c-552 markedly accelerates the reduction of cytochrome c by Na_2S at very low concentrations. The enzymatic activity of the cytochrome could be of physiological significance, since the organism photosynthetically oxidizes sulfide to acquire energy and reducing power for its growth. Although, in general, cytochrome c is rapidly reduced non-enzymatically by sulfide, cytochrome c-552 accelerates the reduction of cytochrome c even when the concentration of sulfide is so low that cytochrome c is not spontaneously reduced. Further, as the organism has to take up electrons into cells from sulfide present in the medium, the biological reduction of the cytochromes or other redox proteins in the cells by sulfide could be different from the chemical reduction which occurs when cytochrome c is

mixed with sulfide in solution. The findings that cytochrome
c-552 is extraordinarily rapidly reduced with sulfide as compared
with other cytochrome c such as horse cytochrome c will support
the idea that the cytochrome is an enzyme, i.e. sulfide-cytochrome
c reductase.

It is of interest to clarify which protein is the electron
acceptor for sulfide oxidation catalyzed by cytochrome c-552 in
vivo. Cytochrome c' or cytochrome c-553(550) purified from
C.vinosum does not act as the electron acceptor in the oxidation
of sulfide by cytochrome c-552. Although a few kinds of C-type
cytochromes exist in the organism in addition to the above proteins,
the quantity in the cells seems to be too small for them to act
as electron acceptors in the active oxidation of sulfide. Yamanaka
and Kusai have reported that a flavocytochrome, cytochrome c-553
derived from C.limicola f. thiosulfatophilum functions in the
oxidation of sulfide and cytochrome c-555 of the organism acts
as the electron acceptor in the oxidation of sulfide. Chromatium
cytochrome c-553(550) resembles C.limicola f. thiosulfatophilum
cytochrome c-555; both cytochromes seem to be c_6-type cytochrome
(17, unpublished data). Therefore, it was expected that cyto-
chrome c-553(550) would act as the electron acceptor for sulfide
oxidation with cytochrome c-552 in C.vinosum just as in C.limicola
f. thiosulfatophilum. However, cytochrome c-553(550) does not
act as the electron acceptor. Morita et al. (5) have suggested
that Chromatium cytochrome c-553(550) may participate in a photo-
synthetic pathway different from that in which cytochrome c-552
functions, and it has been reported by other workers (3,4)

-52-

that Chromatium cytochrome c-552 is implicated in the electron transfer between reduced inorganic sulfur substrate and the photo-reaction center. Further, it has been claimed that the two hemes of cytochrome c-552 can be distinguished by EPR method (18). Therefore, it seems likely that one of the two hemes in the cyto-chrome c-552 molecule plays the same role as Chlorobium cytochrome c-555, so that the electrons from sulfide may be directly trans-ported to the light-excited bacteriochlorophylls in the active centers.

C.vinosum cells accumulate granules of elementary sulfur photosynthitically in cells while sulfide or thiosulfate is pre-sent in the medium. The elementary sulfur thus accumulated is finally oxidized to sulfate (23). Therefore, an oxidized system of elementary sulfur should exist in Chormatium and Chlorobium. However, nothing has been known of the molcular mechanism in the sulfur oxidation in these sulfur photosynthetic bacteria, although it has been found that an oxygenase participates in the oxidation of the sulfur in Thiobacilli (56). It seems very interesting and important to elucidate the oxidation mechanisms of elementary sulfur under the photo-anaeribic conditions in the sulfur photo-synthetic bacteria.

Under the dark-anaerobic conditions, the Chromatiaceae excrete sulfide which originates from the intracellular sulfur globules (24). This has been found with C.vinosum (24,25). Van Gemerden (26,27) has demonstrated that in C.vinosum, there exists a strict stoichio-metric coupling between sulfide formation from sulfur and trans-formation of poly-glucose into poly-β-hydroxybutyrate under the

dark-anaerobic conditions. This means that elemental storage sulfur under these circumstances serves as a sink for electrons; the sulfide is excreted and ATP is formed via glycolysis. Namely, an electron flow from NAD(P)H toward elementary sulfur must be possible. As cytochrome c-552 acts also as a reductase of elementary sulfur as has been found in the present investigation, the cytochrome probably participate in the electron transfer to elementary sulfur, although the direct electron donor for the cytochrome in vivo has not yet been known.
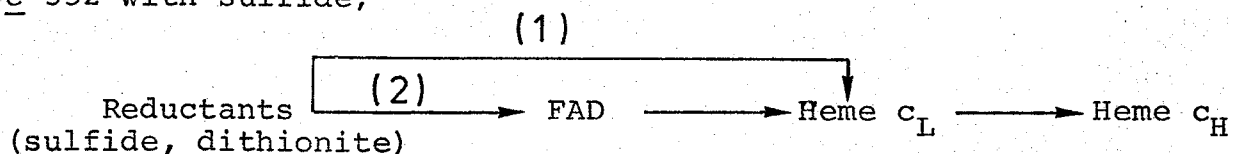
It is necessary to elucidate the order in which an electron is transported between the prosthetic groups in the cytochrome c-552 molecule to clarify the physiological roles of the two hemes and FAD. The reductive titration curve of cytochrome c-552 coincides with the theoretical titration curve with n=1, as shown in Figure 18. This indicates that the two hemes of cytochrome c-552 do not accept electrons at the same time. Namely, it seems probable that electrons transfer sequentially from the low-potential heme c molecule to the high-potential heme c molecule. The change in the absorption spectrum of cytochrome c-552 during its reduction with sulfide, as shown in Figure 20, is the same as that is observed during it reduction with dithionite. Therefore, it seems probable that electrons from sulfide are also transported between two hemes sequentially, although the reductive titration of cytochrome c-552 was performed only with $Na_2S_2O_4$ as sulfide damages the electrode.

Further, it has been found that electron is transported between heme c and flavin in the molecule of Chromatium cytochrome

c-552. Although cytochrome c-552 is slightly reduced in the

presence of EDTA by irradiation with a 100 watt incandescent

lamp for 24 h as shown in Figure 12, cytochrome c-552 is rapidly

photoreduced with EDTA as the photosubstrate in the presence of

free flavins such as FAD or FMN. Massey et al. (46) have demon-

strated that free flavins catalyze very efficiently the phto-

reduction of a wide variety of flavoproteins. The catalytic

effect of flavins is ascribed to their photoreduction followed

by dark reactions between the free dihydroflavin formed and the

flavoproteins. An aged cytochrome c-552 preparation is more

rapidly photoreduced than the fresh one. This is presumably

attributable to the presence of free flavin, which is caused by

hydrolysis of the flavin bound of the cytochrome in the aged
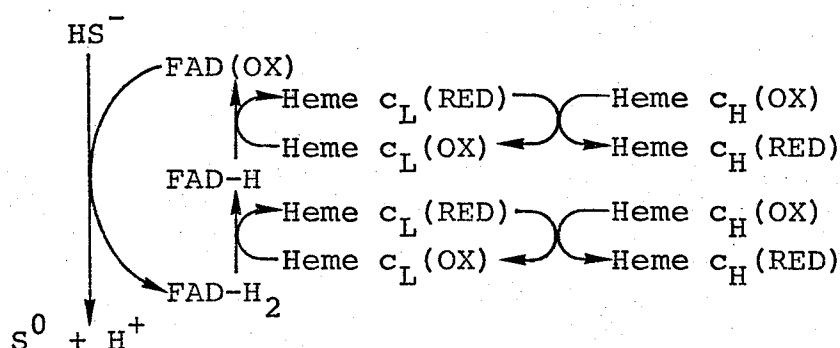
preparation.

When cytochrome c-552 was subjected to laser illumination

in the presence of EDTA for 30 min under anaerobic conditions,

the Raman lines attributable to oxidzed flavin disappear and

those to reduced heme c appear. These findings confirm occurrence

of electron transport from flavin to heme in the molecule of cyto-

chrome c-552, as heme c in the cytochrome is not directly reduced

by the laser illumination under the conditions mentioned above.

On the basis of the findings described above, the following

schemes may be thought for the reduction mechanism of cytochrome

c-552 with sulfide;

$$\text{Reductants} \xrightarrow{\quad (2) \quad} \text{FAD} \xrightarrow{\quad\quad} \text{Heme } c_L \xrightarrow{\quad\quad} \text{Heme } c_H$$
(sulfide, dithionite)

(1)

It is difficult to determine from the static studies whether electrons are transported by the pathway (1) or by the pathway (2). Although kinetic studies should be necessary for elucidation of the question, reduction of cytochrome c-552 with sulfide was too fast to be followed in the stopped-flow apparatus. Meanwhile, cyanide binds to the flavin of cytochrome c-552 (Figure 12) and inhibits the sulfide-cytochrome c reductase activity of the flavocytochrome. These results suggest that electrons from sulfide are transferred to cytochrome c via the flavin of cytochrome c-552. This electron transport sequence in the cytochrome c-552 molecule will be supported by another finding. Yong et al. have concluded on the basis of the ORD of cytochrome c-552 that the two heme molecules and one flavin molecule in the cytochrome molecule are located very closely to one another (15). Further, the sequential electron transport from flavoprotein to cytochrome b in the mitochondrial respiratory chain seems to be favorable to the idea mentioned above about the relatioship in the electron transport between the flavin and hemes of the cytochrome c-552 molecule. On the basis of the findings and discussions mentioned above, it seems difficult to accept the electron transport pathway in the above scheme (1). Therefore, I would like to propose the following scheme for the reduction of cytochrome c-552 with sulfide:

HS^-

FAD(OX)

Heme c_L(RED) —— Heme c_H(OX)

Heme c_L(OX) —— Heme c_H(RED)

FAD-H

Heme c_L(RED) —— Heme c_H(OX)

Heme c_L(OX) —— Heme c_H(RED)

FAD-H$_2$

$S^0 + H^+$

-56-

If the reduction of cytochrome c-552 with sulfide follows the mechanism described above, the semiquinone of flavin would be formed during th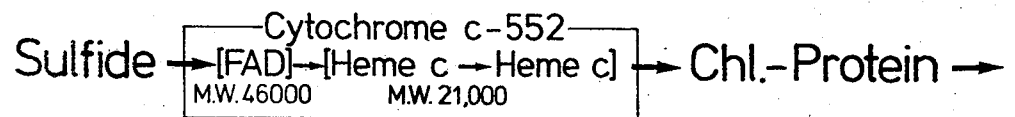e reduction of the cytochrome with sulfide. It is a future problem to examine by ESR if the semiquinone of flavin is formed in the cytochrome c-552 molecule.

It is clear that Chromatium cytochrome c-552 consists of two subunits, a flavoprotein moiety with a molecular weight of 46,000 and a cytochrome moiety with a molecular weight of 21,000. The flavoprotein subunit can be obtained by isoelectric focusing in the presence of 6M urea and 1 % β-mercaptoethanol, while the cytochrome subunit could not be obtained by the same method. This may be attributable to the instability to β-mercaptoethanol of the heme of cytochrome c-552. In the absence of β-mercapto-ethanol, cytochrome c-552 was not completely split into the two moieties. Thus, even in polyacryamide gel electrophoresis three bands were detected in the presence of 6M urea but absence of β-mercaptoethanol, while two bands were detected in the presence of both 1 % β-mercaptoethanol and 6M urea. The excess band observed in the absence of β-mercaptoethanol seems to be due to original cytochrome c-552 which has survived the electrophoretic procedure. Therefore, β-mercaptoethanol is essential to obtain pure flavoprotein subunit. Accordingly, in order to obtain the cytochrome moiety, a milder procedure is necessary where the flavo-protein moiety is not completely separated from the intact flavo-cytochrome c. Attempts to obtain both subunits simultaneously have so far been unsuccessful. However, it appears to be correct that the molecule of cytochrome c-552 is composed of two kinds of

subunits, since N-terminal analysis of the cytochrome has given two amino acid residues, alanine and glutamic acid, while the terminal residues of the cytochrome and flavoprotein moieties has been found to be glutamic acid and alanine, respectively. The molecular weight of cytochrome c-552 is 53,000 as determined by gel filtration on Sephadex G-100 in the present study. This value differs a little from that reported by Bartsch et al. (13). In any case, as judged from the molecular weights of cytochrome c-552 and its two subunits, we can conclude that the molecule of the flavocytochrome is composed of one molecule each of the cytochrome and flavoprotein subunits.

Yamanaka has reported that the flavocytochrome c of C.limicola f. thiosulfatphilum also consists of two subunits, a flavoprotein subunit with a molecular weight of 47,000 and a hemoprotein sub- unit with a molecular weight of 11,000 (51). Flavocytochromes c such as C.limicola f. thiosulfatophilum cytochrome c-553 and C.vinosum cytochrome c-552 are isolated from very limited groups of photosynthetic bacteria which oxidize sulfide photosynthetically. Further, both cytochromes have sulfide-cytochrome c reductase activity and similar molecular features as shown in Figure 28. It will be interesting to study the evolutionary relationship between the two flavocytochromes c , e.g. by comparison of the amino acid sequences, and hence that between Chlorobium and Chromatium.

Chromatium vinosum

Sulfide → [FAD] → [Heme c → Heme c] → Chl.-Protein →

Cytochrome c-552

M.W. 46000   M.W. 21,000

Chlorobium l.f.thiosulfatophilum

Sulfide → [FAD] → [Heme c] → [Heme c] → Chl.-Protein →

Cyt. c-553   Cyt. c-555
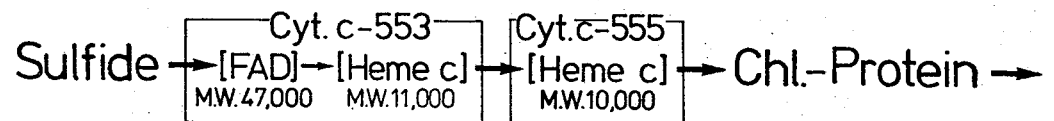
M.W. 47,000   M.W. 11,000   M.W. 10,000

Figure 28. Comparison between Chromatium vinosum and Chlorobium limicola f. thiosulfatophilum with respect to the oxidation of sulfide.

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II. STOPPED-FLOW AND FLASH PHOTOLYSIS STUDIES ON THE REACTION
     OF REDUCED CYTOCHROME C-552 WITH CARBON MONOXIDE

INTRODUCTION

Chromatium _vinosum_ cytochrome c-552 possesses two molecules of heme c and one molecule of covalently bound FAD per molecule (1). Fukumori and Yamanaka have reported that _Chromatium_ cytochrome c-552 acts as sulfide-cytochrome c reductase and probably participates in the photosynthetic oxidation of sulfide in the organism (2). Usually c-type cytochromes do not react with carbon monoxide (CO), and as rare cases _Chromatium_ cytochrome c-552 (1), cytochrome c_3 (3) and cytochrome c' (4) have been reported to react with CO. Bartsch et al. speculated that only one of the two hemes of cytochrome c-552 is involved in the CO-complex formation. In the present investgation, we carried out stopped-flow and flash photolysis measurements on the reaction between reduced _Chromatium_ cytochrome c-552 and CO, and tried to analyze the experimental data based on a proposed reaction scheme. A good agreement between the experimental and calculation data prompted us to propose that the two hemes in cytochrome c-552 react with CO with different affinities and that the CO complex is fairly light sensitive.

MATERIALS AND METHODS

Chromatium _vinosum_ cytochrome c-552 was purified as described by Bartsch and Kamen (1,4), and dissolved in 10 mM Tris-HCl buffer, pH 8.5. The concentration of cytochrome c-552 was determined spectrophotometrically using ε_{mM} of 54 at 552 nm (1). To prepare CO-cytochrome c-552, the cytochrome was reduced with a small amount of sodium dithionite and the solution was bubbled with CO gas.

CO gas was obtained commercially and used without further purification. Absorption spectra were recorded on a computer-controlled single beam spectrophotometer constructed of Union Giken spectrophotometer modules. The whole machine was run by a microcomputer, system 71. Kinetic studies were conducted on a Union stopped-flow rapid scan analyzer, model RA-601, equipped with flash-photolysis accessories. Data analyses were made on a Union microcomputer system 77. The temperature was maintained at 25^0C, unless otherwise stated, by use of a Haake K and Fw and it was monitored with a thermister.

RESULTS AND DISCUSSION

A Carbon Monoxide Complex of Chromatium cytochrome c-552

Chromatium vinosum cytochrome c-552 formed a carbon monoxide complex in the reduced state. Upon the complex formation the Soret peak at 416 nm of the reduced cytochrome shifted to 414 nm accompanying a increase in absorbance (Figure 1-A) in accordance with the original observation by Bartsch et al. (1). A difference spectrum of the CO complex versus the reduced cytochrome showed a peak and a trough at 415 and 425 nm, respectively, as well as a small peak at 394 nm (Figure 1-B). In the visible region the α- and β-peaks diminished in intensity and a difference spectrum was characterized by peaks at 566 and 539 nm and troughs at 554 and 522 nm (Figure 2); that is, carbon monoxide displaced these peaks towards red contrary to the blue shift of the Soret peak.

When a solution of CO-cytochrome c-552 was bubbled through with argon gas,the reduced form was restored.
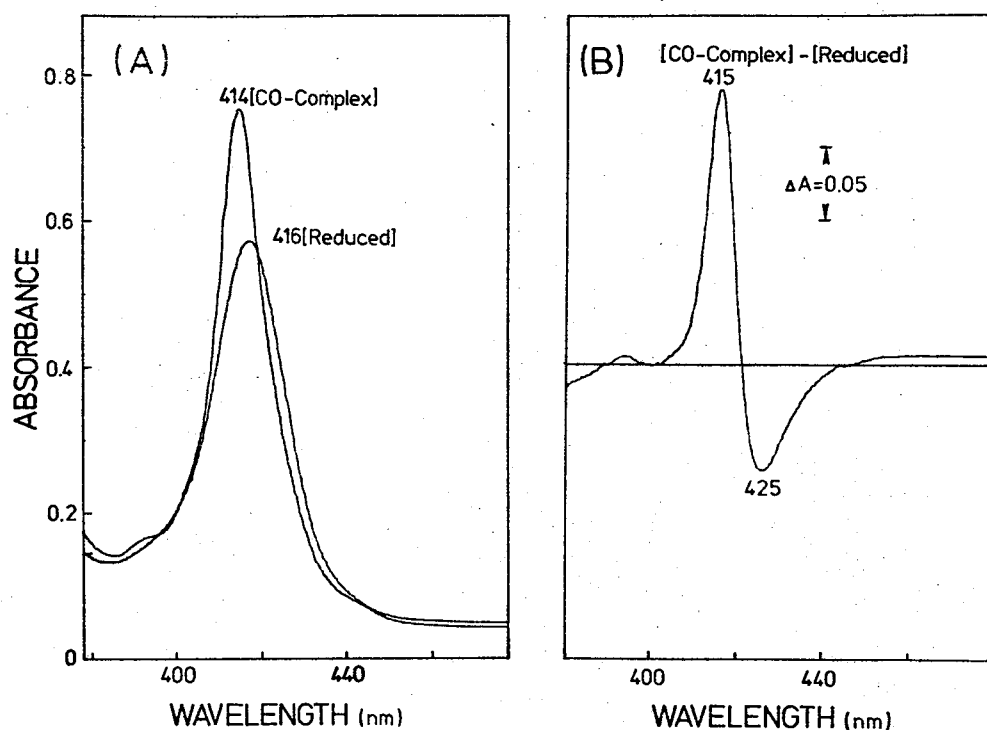
-65-

Figure 1.   (A) Absorption spectra of reduced cytochrome c-552
(1.4 μM) and CO-cytochrome c-552.   Cytochrome c-552 was dissolved
in 10 mM Tris-HCl buffer, pH 8.5, and reduced with a small amount
of sodium dithionite.   CO-cytochrome c-552 complex was prepared
by the following method.   After cytochrome c-552 was completely
reduced with dithionite, CO gas was bubbled through the solution
for a few minutes.

(B) A difference spectrum between CO-cytochrome c-552 and the reduced
cytochrome in the Soret region.   Reduced cytochrome c-552 (1.4 μM)
and CO-cytochrome c-552 were prepared by the method as described
above.
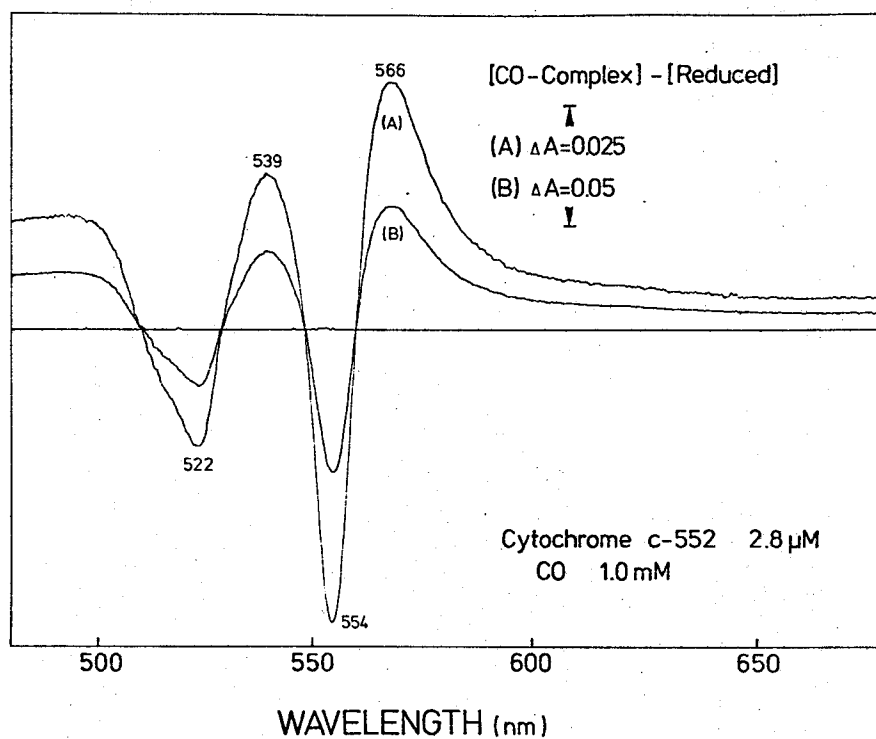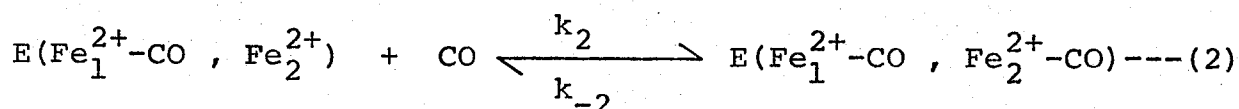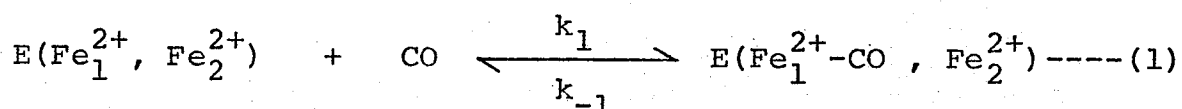
Figure 2. Difference spectra between CO-cytochrome c-552 and the reduced cytochrome in the visible region. Reduced cytochrome c-552 (2.8 μM) and CO-cytochrome c-552 were prepared by the method as described in the legend to Figure 1.

Stopped-Flow Experiments on the Reaction of Reduced Cytochrome c-552 with Carbon Monoxide

When the reaction of reduced Chromatium cytochrome c-552 with CO was followed on a stopped-flow apparatus, an absorbance change did not follow an apparent first order kinetics (Figure 3). A semilogarithmic plot of the absorbance change due to a decrease in the concentration of the reduced cytochrome apparently was biphasic in contrast with the result obtained by using bovine heart cytochrome oxidase (5), in which the reaction proceeded monophasically. As Chromatium cytochrome c-552 contains two c-type hemes in a molecule, it is speculated easily that both of them react with CO but in different manners. Therefore, we attempted to explain the observed results based on the simplest reaction scheme as follows,

$$E(Fe_1^{2+}, Fe_2^{2+}) + CO \xrightarrow[k_{-1}]{k_1} E(Fe_1^{2+}\text{-}CO, Fe_2^{2+}) \text{----(1)}$$

$$E(Fe_1^{2+}\text{-}CO, Fe_2^{2+}) + CO \xrightarrow[k_{-2}]{k_2} E(Fe_1^{2+}\text{-}CO, Fe_2^{2+}\text{-}CO) \text{---(2)}$$

where Fe_1^{2+} and Fe_2^{2+} signify each of the two hemes. At an infinite time after initiation of the reaction equilibrium will established among E, E·CO, and E·(CO)$_2$; in the meantime a decrease of E with time is expressed by

$$[E]_t = [E]_0 (C_0 + C_1 \exp(-k't) + C_2 \exp(-k''t)) \text{---------(3)}$$

where $[E]_t$ and $[E]_0$ are the concentration of reduced Chromatium cytochrome c-552 at time = t and 0, respectively, and
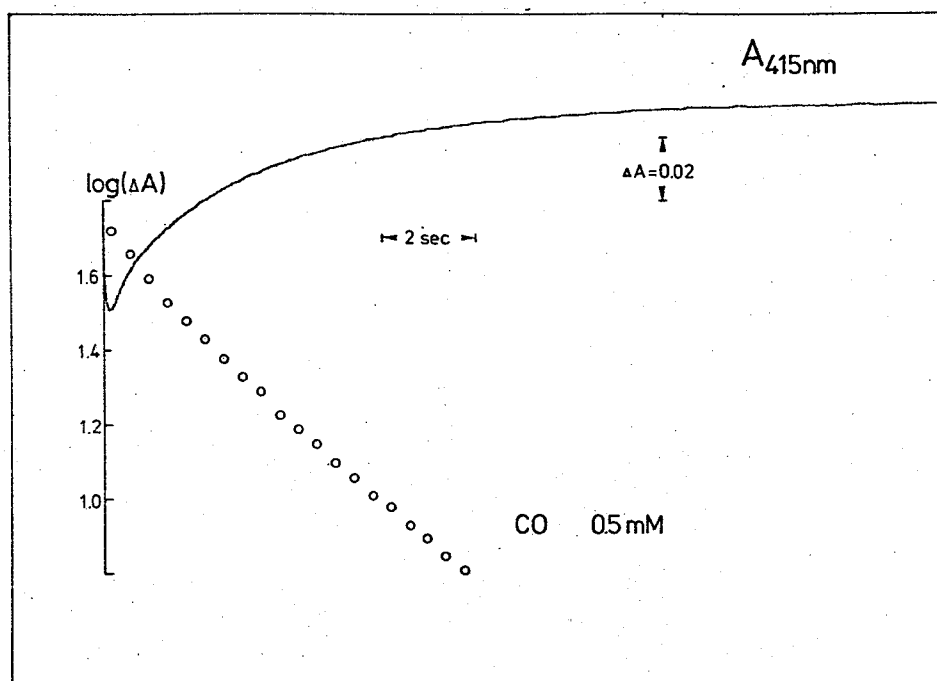
Figure 3. The absorbance change of reduced cytochrome c-552
after mixing with CO. Reduced cytochrome c-552 was dissolved
in 10 mM Tris-HCl buffer, pH 8.5, and placed in one of the reser-
voires of a stopped-flow apparatus. CO saturated buffer containing
a small amount of sodium dithionite was placed in the other reser-
voir. The two solutions of an equal volume were drived into a
mixing chamber under a pressure of 5 kg/cm^2. The absorbance change
was followed at 415 nm. The concentration of cytochrome c-552
in the reaction mixture were 2.8 μM and temperature 25°C.

$$C_0 = \frac{k_{-1}k_{-2}}{\beta} \qquad C_1 , C_2 = \text{constant} \quad \text{--------(4)}$$

$$k' = \alpha + \sqrt{\alpha^2 - \beta} \quad \text{----(5)} \qquad k'' = \alpha - \sqrt{\alpha^2 - \beta} \quad \text{-----(6)}$$

α and β are functions of k_1, k_{-1}, k_2, k_{-2} and a concentration of CO, [CO], as follows:

$$\alpha = \frac{1}{2}(k_1[CO] + k_{-1} + k_2[CO] + k_{-2}) \quad \text{----------------(7)}$$

$$\beta = k_1k_2[CO]^2 + k_1k_{-1}[CO] + k_{-1}k_2[CO] \quad \text{------------(8)}$$

If assume that [CO] is sufficiently high enough to convert the reduced cytochrome into the CO complex completely, k' and k" will become

$$k' = k_1[CO] + k_{-1} \quad \text{------------------------(9)}$$

$$k'' = k_2[CO] + k_{-2} \quad \text{------------------------(10)}$$

and C_0 can be regarded to be 0. Consequently, an absorbance change after initiation of the reaction is expressed by the following equation if a fractional absorbance change due to $E \longrightarrow E \cdot CO$ is defined as R,

$$\Delta A_t = \Delta A_\infty [\ 1 + (R \cdot k' - k'')/(k'' - k') \exp(-k't) + (1 - R)k'/(k''-k')$$
$$\exp(-k''t)\ ] \quad \text{------------------------------(11)}$$

where ΔA_∞ is the total absorbance change determined experimentally.

The stopped-flow apparatus which we employed in the present study was designed to allow mixing of two solutions only in an equal volume, accordingly a maximal concentration of CO after mixing was not higher than 0.5 mM. Although this concentration turned out not to be sufficient to saturate all of the reduced hemes, as will be shown by subsequent experiments, we tentatively supposed that this would satisfy the above requirement. Figure 4 indicates clearly that an agreement between the experimental
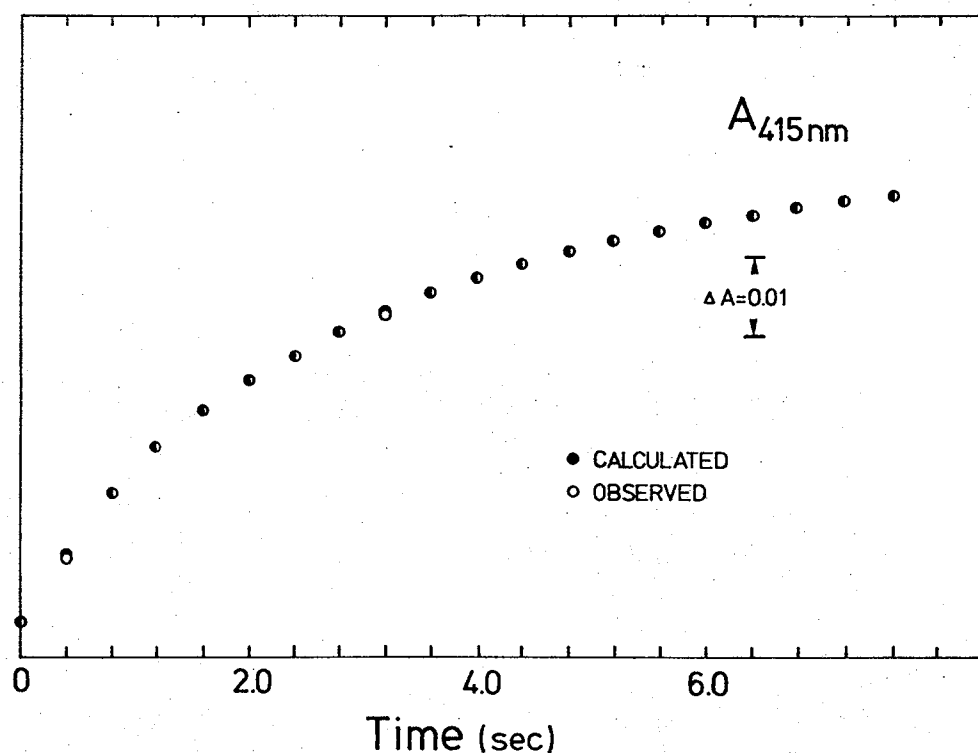
Figure 4. Calculated absorbance changes of reduced cytochrome c-552 after mixing with CO. The experimental data in Figure 3 were compared with computer generated points. The calculated curve which best fitted with the experimental data was obtained by assuming that the absorbance change at 415 nm obeyed the equation (11) (see "RESULTS AND DISCUSSION"). The best fitted curve was obtained by using the following values for k'', k', and R: $k'' = 0.24$ sec^{-1}, $k' = 0.83$ sec^{-1}, and R = 0.5.

(o), Experimental data; (o), calculated values.

and calculation data is satisfactory. The theoretical curve was obtained by using $k'=0.83$ sec^{-1} and $k''=0.24$ sec^{-1}, and the best fit was obtained when $R = 0.5$. The experimental results that were obtained at different concentrations of cytochrome $\underline{c}$-552 between 0.7 and 2.8 μM were also analyzed in the same way, giving the same kinetic parameters as illustrated in Figure 5. On the contrary, when the reactions were carried out in the presence of CO lower than 0.5 mM, the experimental data could not be explained by Equation (11), probably because the requirement for the carbon monoxide concentration did not hold any more in a practical sense. The agreement between the experiment and theory strongly indicates that both heme $\underline{c}$'s in $\underline{Chromatium}$ cytochrome $\underline{c}$-552 react with CO and that the extent of a spectral change for conversion of E to E·CO is half that for a change of E to E·$(CO)_2$. In accordance with this, the absorption spectra recorded by rapid scanning spectrophotometer during the CO complex formation did not show any sign for an appearance of any new spectral species.

In order to determine the equilibrium constants K_1 and K_2 for the reactions expressed by equations (1) and (2) it is neceaary to know the concentrations of E, E·CO, and E·$(CO)_2$ at different concentrations of CO. When reduced $\underline{Chromatium}$ cytochrome $\underline{c}$-552 was mixed with an equal volume of a solution containing CO on a stopped flow apparatus, an absorbance change approached a certain level depending on the concentrations of CO. The change was completed within 30 sec even when the CO concentration was 0.1 mM, the lowest ever used. This result indicates that equilibrium was attained in this time range. The extent
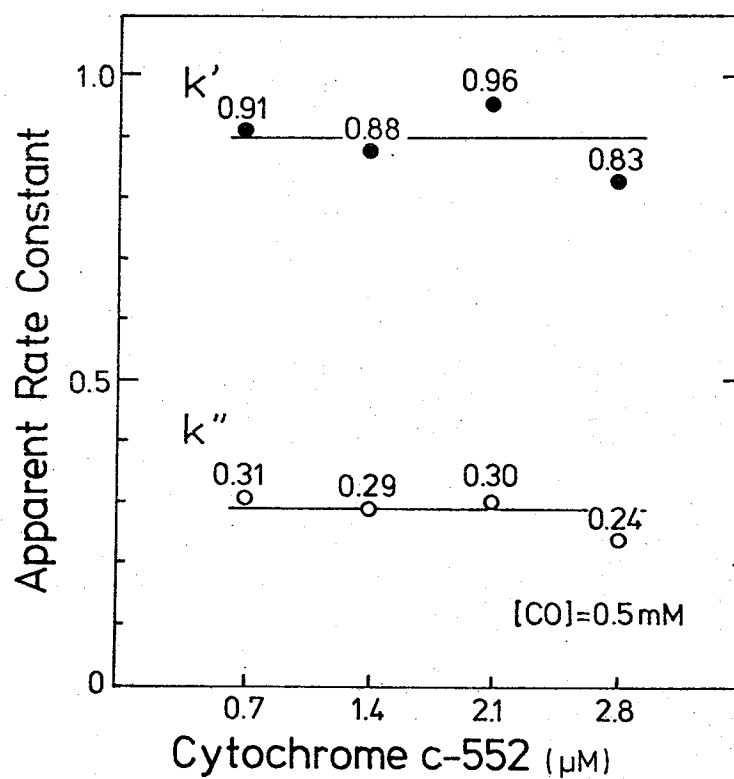
Figure 5. Dependence of k' and k" on the concentration of cyto-
chrome c-552. The experimental conditions were as described in
the legend to Figure 3, except that the concentration of cytochrome
c-552 was changed between 0.7 μM and 2.8 μM. The values of k' and
k" were obtained by the method as described in the text.

of the total absorbance change due to complete conversion of E

into $E \cdot (CO)_2$ was estimated by extrapolating a plot of $1/\Delta OD$

against $1/[CO]$ to $1/[CO] = 0$. Consequently, the relationship

among a fractional absorbance change and the concentrations of

E, $E \cdot CO$, and $E \cdot (CO)_2$ is expressed by

$$F = \frac{[E(CO)] + 2[E(CO)_2]}{2[E]_t} \quad \text{---------------------(12)}$$

and

$$[E]_t = [E] + [E(CO)] + [E(CO)_2] \quad \text{-----------(13)}$$

The coefficient of 2 in equation (12) was derived from R=0.5.

Then equation (12) leads to

$$F = \frac{K_2[CO] + 2[CO]^2}{2(K_1 k_2 + K_2[CO] + [CO]^2)} \quad \text{--------------(14)}$$

This is transformed into

$$\frac{2[CO](1 - F)}{(2F - 1)} = K_2 + K_1 K_2 \cdot \frac{2F}{[CO](2F - 1)}$$

At various carbon monoxide concentrations the corresponding

values of F can be obtained by calculation. A plot of $2[CO](1-F)/$

$(2F-1)$ against $2F/([CO] \cdot (2F-1))$ then would give a straight line

with an intercept of K_2 and a slope of $K_1 K_2$. The values of K_1

and K_2 thus determined were 2.4×10^{-4}M and 4.2×10^{-4}M, respectively.

Using these values and equation (14), a theoretical curve was

obtained fitting satisfactorily well with the experimental values

(Figure 6). This agreement further supports the previous proposal

that the two heme c molecules react with CO. The kinetic para-

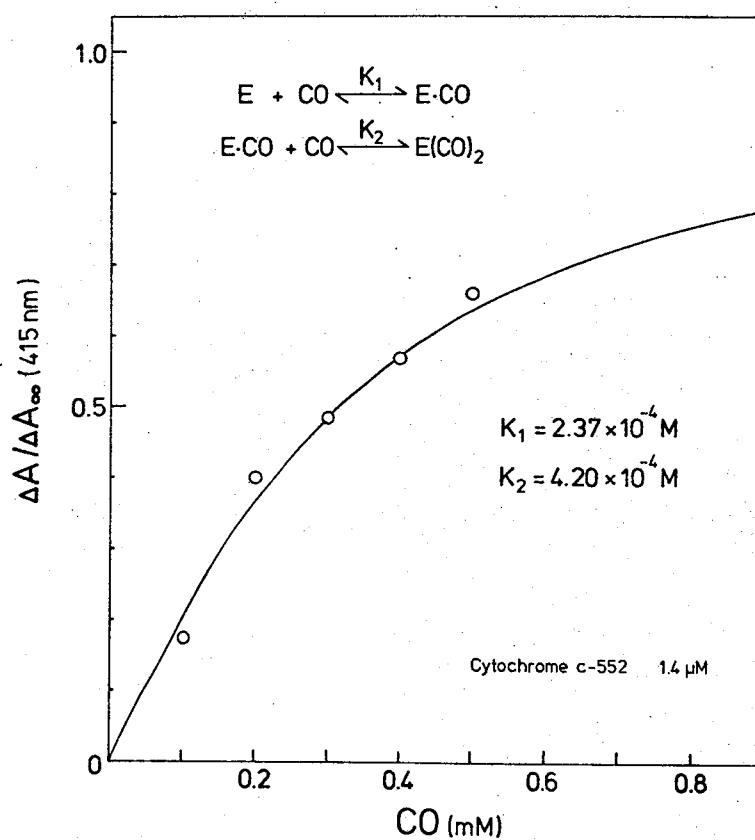meters in equations (1) and (2) were calculated as follows:

Figure 6. The effect of CO concentration on the extent of an absorbance change at 415 nm on reaction of reduced cytochrome c-552 with CO. The experimental conditions were as described in the legend to Figure 3, except the concentration of CO.

$k_1 = 1.2 \times 10^3 M^{-1} sec^{-1}$, $k_2 = 3.2 \times 10^2 M^{-1} sec^{-1}$, $k_{-1} = 0.29\ sec^{-1}$ and $k_{-2} = 0.13\ sec^{-1}$.

It is also noteworthy that the extent of saturation of heme $\underline{c}$ in $\underline{Chromatium}$ cytochrome $\underline{c}$-552 with CO was 0.65 at 0.5 mM CO and 0.8 even at 1 mM CO.

Photochemical Properties of CO-Chromatium cytochrome c-552

A flash photolysis apparatus employed in the present study was designed so that a monitoring light from the source entered a sample cuvette through a neutral filter and the transmitted light was dispersed in the monochrometer. Consequently, when the intensity of the monitoring light was strong, little absorbance change was observed after firing of a flash tube. However, by diminishing the light intensity the photodissociation was observed spectrophotometrically. Since the photodissociation of a carbon monoxide complex of mammalian cytochrome oxidase did not occur appreciably by a monitor light under a similar condition, it was concluded that the CO complex of $\underline{Chromatium}$ cytochrome $\underline{c}$-552 is fairly light sensitive. In fact, even a monochromatic light incident on the CO complex was found to photodissociate the sample especially when the light intencity was strong. Further photodissociation was achived by illuminating the sample cuvette at a right angle to the monitor beam.

The recombination process of CO following the photodissociation was completed usually within 10 seconds at different temperatures examined (Figure 7). This process was biphasic and again contrasted with the monophasic recombination process observed with mammalian cytochrome oxidase. However, difference spectra obtained during this
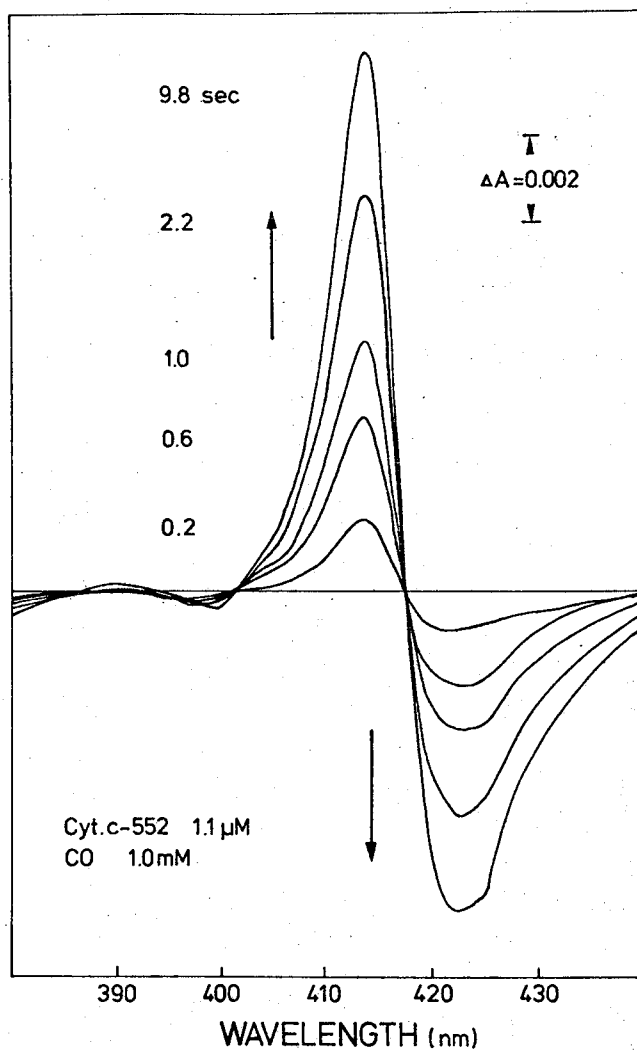
Figure 7. Spectral changes during the reaction of reduced cyto-
chrome c-552 with CO. Reduced cytochrome c-552 (1.1 μM) was
dissolved in 10 mM Tris-HCl buffer, pH 8.5, saturated with CO.
After the sample was irradiated with a flashtube, the absorbance
change at a wavelength between 380 and 440 nm was followed on the
stopped-flow apparatus equipped with the flash-photolysis accesories.

change suggested the occurrence of only two spectral species.
The extent of an absorbance change by photodissociation increased
appreciably as the temperature was increased, although no explana-
tion for this phenomenon was made at the present time. This pheno-
menon was not observed with mammalian cytochrome oxidase. By using
apparent first order reaction constants obtained for the initial
changes at different temperatures, a straight Arrhenius plot was
obtained as illustrated in Figure 8, giving the activation energy
of 4.8 Kcal·mol^{-1}. This value was comparable to that for the
recombination of CO with a hot type of mammalian cytochrome oxi-
dase, 5.3 Kcal·mol^{-1} (5). An absorbance change during the recom-
bination process at 25^{0}C was also analyzed as was done previously
for the stopped-flow data. The best fit was obtained when R=0.5,
and k' and k" were 3.96 sec^{-1} and 1.42 sec^{-1}, respectively.
These values were larger than the corresponding values of k'=0.9
sec^{-1} and k"=0.29 sec^{-1} (Figure 6). In the stopped-flow experi-
ments a part of the increase can be explained by an increase in
the carbon monoxide concentration in the reaction mixture (0.5 mM
$\longrightarrow$ 1.0 mM), although only this factor does not explain the increase.
Therefore, it is conceivable that k_i and k_{-i} also increased re-
flecting a change in the intensity of a monitor light, because
the flash photolysis and stopped-flow apparatus had different
instrumental geometries. Consequently K_1 and K_2 would become
larger as the light intensity is increased, whereas they would
become smaller as the light intensity is diminished. At any rate,
it is apparent that equation (11) also holds for the flash photo-
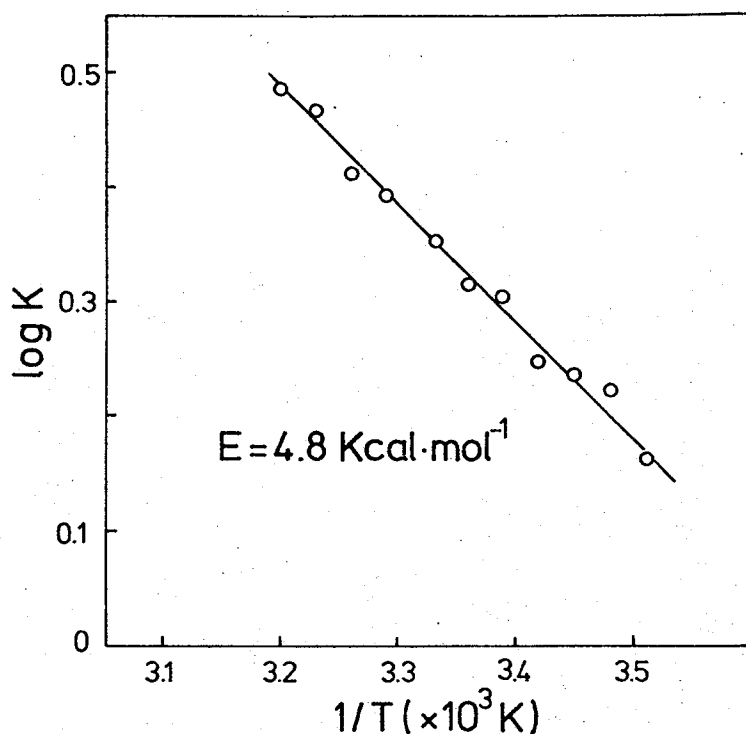lysis data in accordance with the previous proposal.

Figure 8. An Arrhenius plot for the CO-cytochrome c-552 complex formation. The experimental conditions were as described in the legend to Figure 7 except that the concentration of cytochrome c-552 was 2.8 μM. The absorbance change was followed at 415 nm. Temperature was raised stepwise and it was kept constant during measurement. The apparent first-order rate constants were obtained by the method as described in "RESULTS AND DISCUSSION".

Bartsch et al. noticed that an extinction coefficient in the Soret region for a carbon monoxide complex of Chromatium cytochrome c-552 was smaller than the value that was expected for the complexing of the two hemes in that cytochrome with CO, and speculated that only one of them reacted with CO. However, as we described in the preceding section, even 1 mM CO was not high enough to saturate all of the hemes and, furthermore, the extent of the CO complex formation was diminished to a certain extent by an incident monitoring light on the sample. Thus, it is highly probable that the diminished Soret peak of the CO complex observed by them is only apparent due to an incomplete complex formation, their proposal being rendered untenable. In conclusion, we would like to propose, alternatively, that both heme c's in Chromatium cytochrome c-552 combine with CO with different affinities, although the difference is small.

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III. A HIGH POTENTIAL NONHEME IRON PROTEIN-LINKED THIOSULFATE-
OXIDIZING ENZYME DERIVED FROM CHROMATIUM VINOSUM

INTRODUCTION

Chromatium vinosum acquires energy necessary for its processes by photosynthetic oxidation of sulfide and thiosulfate (1). Therefore, photosynthetic oxidation pathways of sulfide and thiosulfate should occur in the organism. Our previous studies have revealed that cytochrome c-552 of the organism acts as sulfide-cytochrome c reductase and probably participates in the photosynthetic oxidation of the salt in the organism (2-4). Van Niel (5) established the overall stoichiometry in thiosulfate oxidation by Chromatium vinosum:

$$2Na_2S_2O_3 \; + \; 4CO_2 \longrightarrow 2H_2SO_4 \; + \; 2Na_2SO_4 \; + \; 4(H_2O)$$
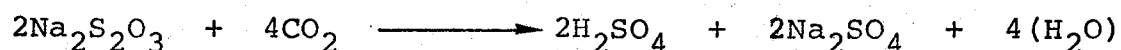
As for the oxidation of thiosulfate in Chromatium, three mechanisms as shown in Figure 1 have been reported: (I) reductive cleavage (6), (II) cleavage by an enzyme having the rhodanese activity (7), (III) oxidation by withdrawing electrons (8). However, details of the enzymatic mechanisms have not been known in either case. Further, our attempts to find the rhodanese activity in the cell free-extracts of C.vinosum have so far been unsuccessful.

Previously, Kusai and Yamanaka have purified from Chlorobium limicola f. thiosulfatophilum a thiosulfate-oxidizing enzyme which is classified in the third group as mentioned above (2). As Chlorobium and Chromatium resemble physiologically each other (1), it is expected that such a thiosulfate-oxidizing enzyme as obtained from C.limicola f. thiosulfatophilum participates in thiosulfate oxidation also in C.vinosum.

In the present investgation, a thiosulfate-oxidizing enzyme has been partially purified and its reaction with various electron acceptors studied. It has been found that HiPIP(high potential nonheme iron protein) of the organism acts as good electron acceptor for the enzyme in thiosulfate oxidation.

MATERIALS AND METHODS

Special reagents

Cytochrome c-552 (9), cytochrome c' (9), cytochrome c-553 (550) (10) and HiPIP (11) were purified by the methods established by Kamen and Bartsch. Tuna cytochrome c was purified by the method of Hagihara et al. (12), and yeast (Saccharomyces oviformis) cytochrome c was kindly supplied by Sankyo Co. Ltd. (Tokyo,Japan).

Cultures

A strain of Chromatium vinosum was kindly supplied by Drs. R.G.Bartsch and T.Meyer (University of California, San Diego, U.S.A.) and large-scale cultivation of the organism was performed as described Bartsch and Kamen (9). Cells were harvested after growth for 5 days, and stored at -20^0C before use.

Polyacrylamide gel electrophoresis

The electrophoresis was performed with slab gel prepared from 7.5 % acrylamide in the absence of sodium dodecyl sulfate. After the electrophoresis had been carried out for 2 h at 20 mA and 150 volt, the salb gel was cut into three strips, and these were separately stained by Coomassie brilliant blue, by the heme-staining reagents (13), and by the use of ferricyanide reduction activity (14).

Enzyme assay

The standard reaction mixture contained 10 mM phosphate buffer, pH 6.2, 0.33 mM $Na_2S_2O_3$, 10-35 μM electron acceptor, and 22 μg enzyme in a total volume of 3.0 ml. When ferricyanide was used as the electron acceptor, its concentration was 0.3 mM. After the enzyme was added to the reaction mixture, the reduction of the electron acceptors was followed spectrophotometrically with time.

Enzyme preparation

The cells (about 100 g in wet weight) were suspended in 200 ml of 10 mM Tis-HCl buffer, pH 8.5, the resulting suspension was homogenized, and treated with sonic oscillator (20 kc, 500 watts; Blackstone, U.S.A.). After allowed to stand for 1 h, the suspension was centrifuged at 10,000 x g for 30 min to remove cell debris. The supernatant obtained here was centrifuged at 100,000 x g for 60 min, and the resulting supernatant was dialyzed for 2 days against 10 mM Tris-HCl buffer, pH 8.5 with several changes of the outer solution. The dialyzed extract was fractionated with $(NH_4)_2SO_4$ and the precipitate formed between 30 % and 50 % saturation was collected by centrifugation at 10,000 x g for 30 min and dissolved in a minimal volume of 10 mM Tris-HCl buffer, pH 8.5. The resulting solution was dialyzed against 0.1 M Tris-HCl buffer, pH 8.5, and charged on a DEAE-cellulose column which had been equilibrated with the same buffer as used for the dialysis. The enzyme was adsorbed on the column. After the column had been washed with 0.1 M Tris-HCl buffer, pH 8.5, the enzyme was eluted by the linear gradient solution which was produced from 300 ml each of 0.1 M Tris-HCl buffer, pH 8.5 containing 0.5 M NaCl. The eluate obtained

with 0.15 to 0.20 M NaCl had the activity to oxidize thiosulfate. By repeating the chromatography with DEAE-cellulose column, a very active enzyme preparation was obtained although it purity was still low as checked by polyacrylamide gel electrophoresis. The partially purified preparation was used to determine the reaction mechanism of the emzyme.

RESULTS

The enzyme preparation was still crude as checked by poly-acrylamide gel electrophoresis. However, the enzyme did not contain heme; when the electrophoresis was performed at pH 4.0, the enzyme moved to anode while the heme protein moved to cathode (Figure 2). Therefore, it is improbable that some cytochrome present as a contaminant in the present preparation functions as a mediator in the oxidation of $Na_2S_2O_3$. The enzyme preparation reduced rapidly HiPIP and cytochromes c of yeast and tuna in the presence of $Na_2S_2O_3$. Figure 3 shows reduction of HiPIP with $Na_2S_2O_3$ in the presence of the thiosulfate-oxidizing enzyme.

The reduction of HiPIP was increased with increase of phosphate concentration until the salt concentration reached about 80 mM, while that of tuna cytochrome c was decreased rapidly with increase of the salt concentration (Figure 4). In the case of yeast cytochrome c, the reaction rate was first increased with increase of the salt concentration and then decreased with further increase of the salt concentration. Horse cytochrome c was poor electron acceptor for the enzyme even in 5 mM phosphate buffer. Ferricyanide was good electron acceptor, and the dependency of

1. $S_2O_3^{2-} + 2e \longrightarrow S^{2-} + SO_3^{2-}$ Hashwa & Pfennig
(1972)

2. $S_2O_3^{2-} + CN^- \longrightarrow SCN^- + SO_3^{2-}$ Smith & Lascelles
(1966)

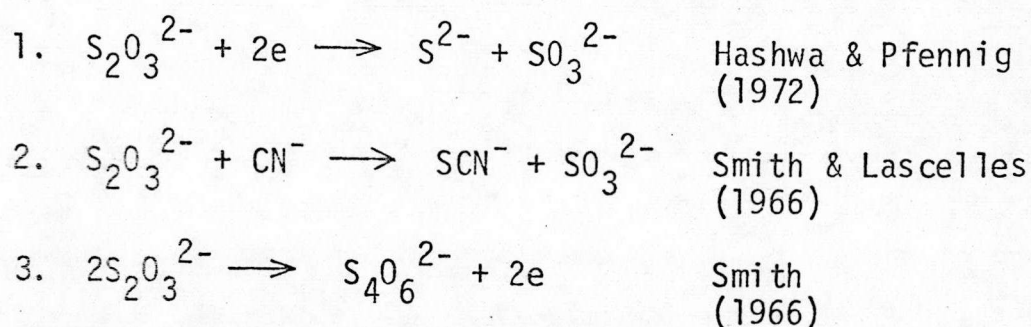3. $2S_2O_3^{2-} \longrightarrow S_4O_6^{2-} + 2e$ Smith
(1966)

Figure 1. Three mechanisms proposed for the oxidation of thio-sulfate in Chromatium vinosum. (1) Reductive cleavage, (2) cleavage by an enzyme having the rhodanese activity, (3) oxidation by with-drawing electrons.
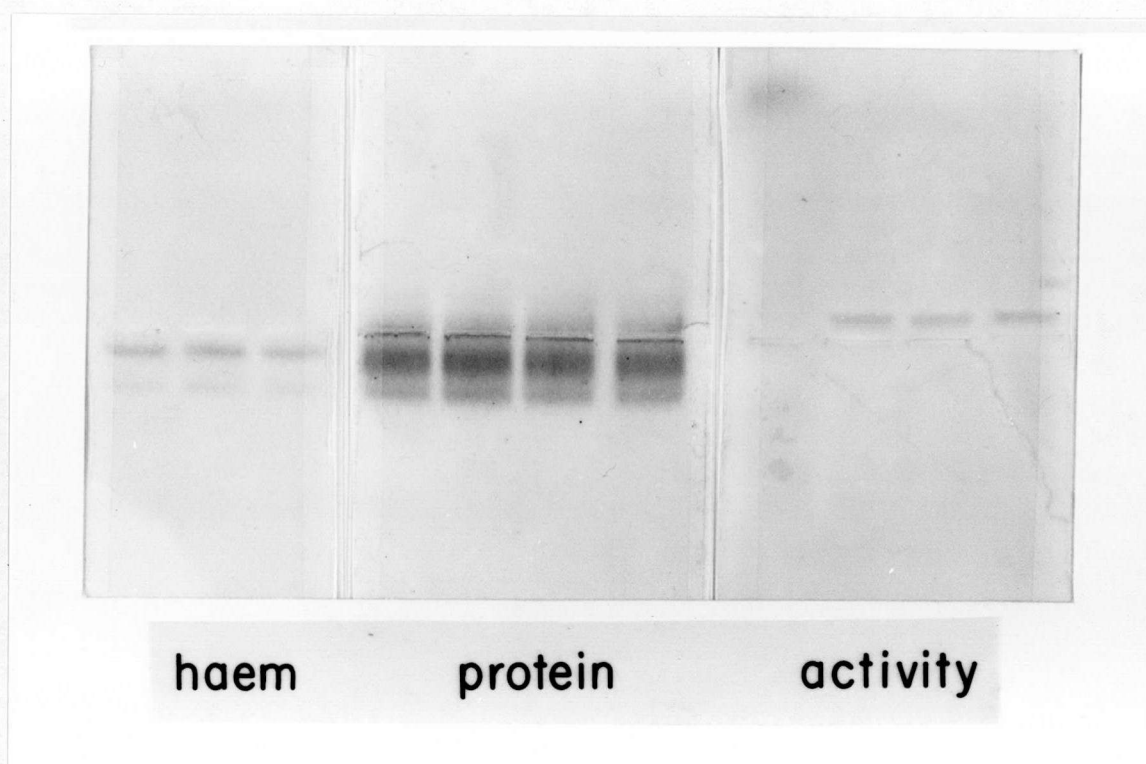

Figure 2. Acrylamide gel electrophoretic profiles of the thio-sulfate-oxidizing enzyme preparation obtained from C.vinosum. The gel was prepared from 7.5 % acrylamide. The electrophoresis was performed in 0.1 M citrate buffer, pH 4.0, for 2 h at 20 mA and 150 volt, and at 4°C.
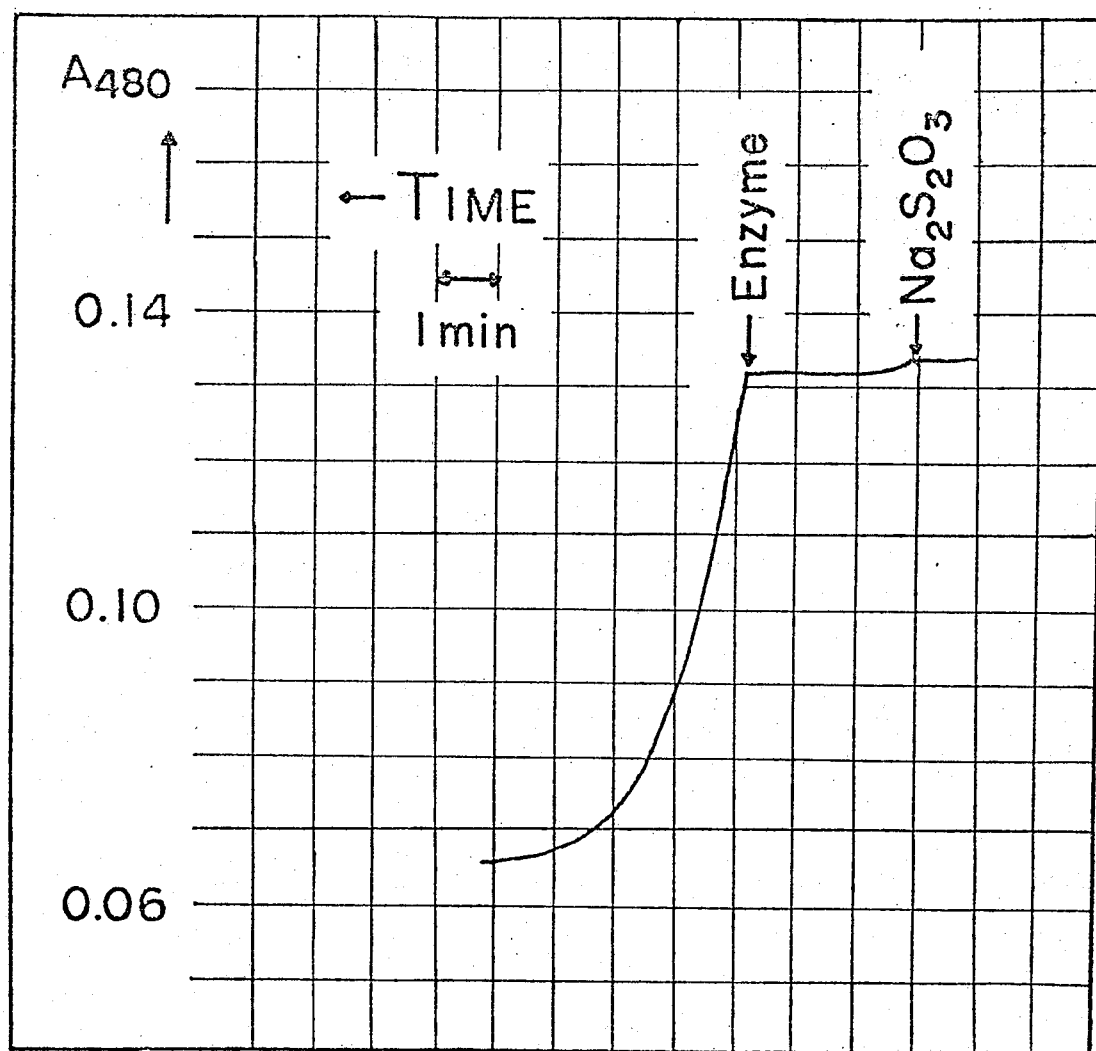
Figure 3. Time course of reduction of HiPIP by the thiosulfate-oxidizing enzyme with $Na_2S_2O_3$. The reaction mixture contained 50 mM sodium-phosphate buffer, pH 6.1, 10 μM HiPIP, 1.7 mM $Na_2S_2O_3$, and 22 μg of the enzyme.
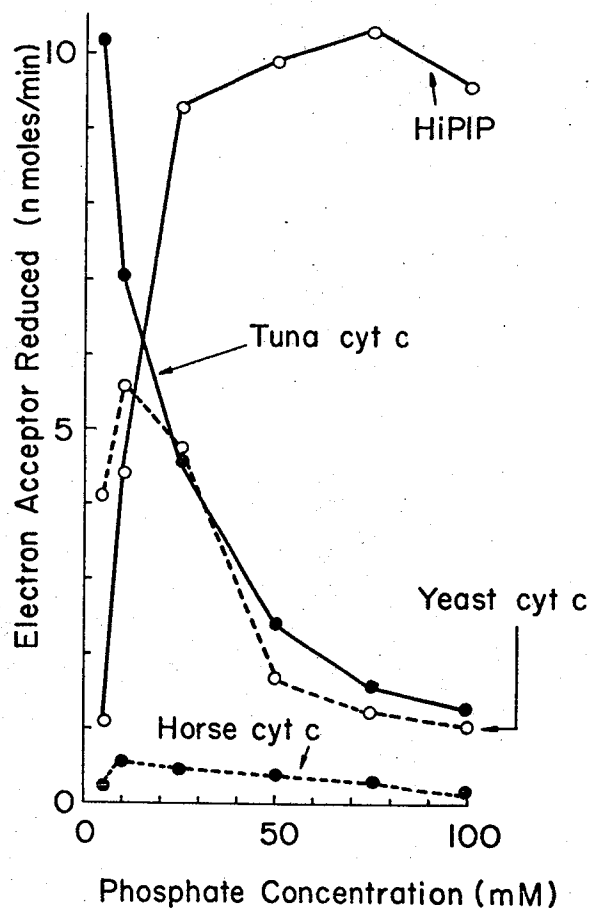
Figure 4. Dependency on phosphate concentration of reduction rates of various electron acceptors catalyzed by C. vinosum thiosulfate-oxidizing enzyme. The concentrations of thiosulfate, HiPIP, yeast cytochrome $\underline{c}$, tuna cytochrome $\underline{c}$, and horse cytochrome $\underline{c}$ were 330 µM, 9 µM, 10 µM and 10 µM, respectively. The amount of the enzyme preparation used for each reaction was 20 µg. A total volume of the reaction mixture was 1.0 ml and the reactions were performed at pH 6.2.

its reduction rate catalyzed by the enzyme was similar to that of the reduction rate of HiPIP as shown in Figure 5. Cytochrome c-552, cytochrome c-553(550) or cytochrome c' derived from C.vinosum did not act as the electron acceptor for the enzyme (Table I). Km values of the enzyme were determined to be 130 μM for HiPIP (Figure 6) and 3.3 mM for ferricyanide. As shown in Table II, the enzyme was 50 % inhibited by 1.3 mM cyanide and 1.7 mM sulfite. This means that the enzyme differs completely from rhodanese. When the enzyme was heated at 100°C for 1 min, it lost the activity completely.

Table III shows the stoichiometry in the reduction of ferricyanide catalyzed by the enzyme in the presence of limited amounts of thiosulfate. In this experiment, ferricyanide was added in excess of thiosulfate. The ratio of ferricyanide reduced to thiosulfate added was unity. From the result, it seems likely that thiosulfate was oxidized to tetrathionate by the enzyme; $2S_2O_3^{2-}$ + $2Fe(CN)^{3-} \longrightarrow S_4O_6^{2-} + 2Fe(CN)^{4-}$. Both sulfite and sulfate as the product in the reaction were not detected by the qualitative analysis (19).

DISCUSSION

The present work has revealed that in C.vinosum the thiosulfate-oxidizing enzyme occurs which resembles the enzyme derived from C.limicola f. thiosulfatophilum (2). One of the differences between the two enzymes is that the Chlorobium enzyme utilizes cytochrome c-555 as the electron acceptor, while the Chromatium enzyme does not react with cytochrome c-553(550).
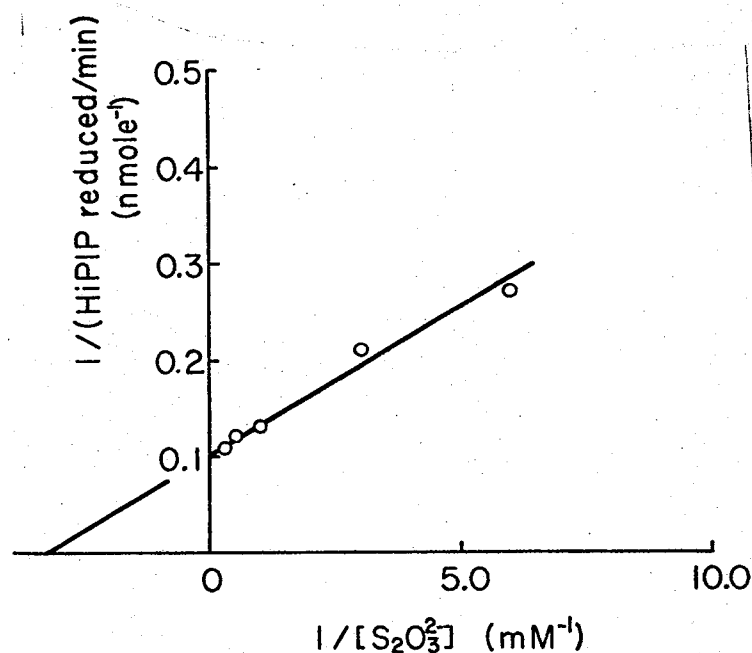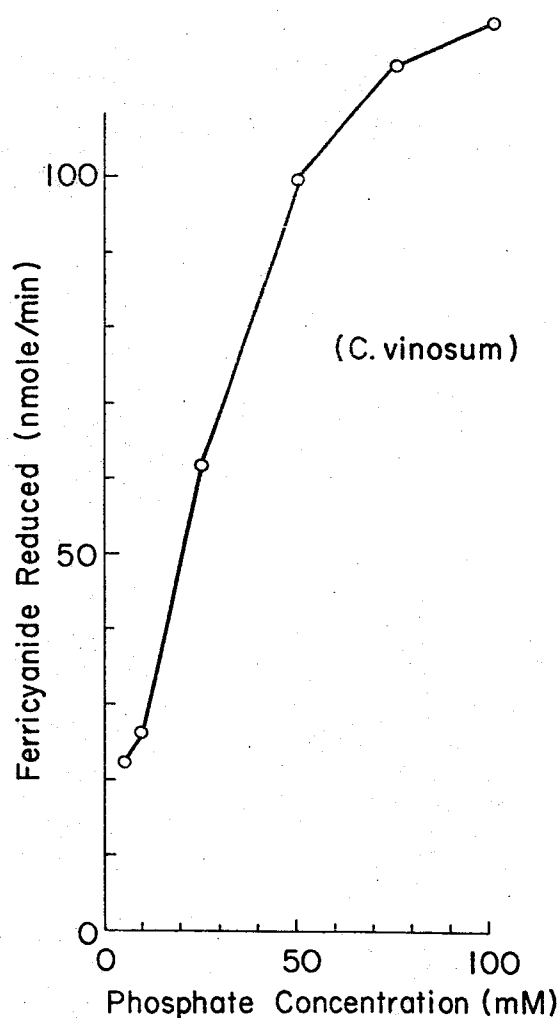
Figure 5. (left)   Dependency on phosphate concentration of reduction rate of ferricyanide catalyzed by C.vinosum thiosulfate-oxidizing enzyme.   The reaction mixture contained 0.3 mM ferricyanide, 330 μM thiosulfate, and 20 μg enzyme preparation.

Figure 6. (right)   Lineveaver-Burk plot for the reduction of HiPIP with thiosulfate catalyzed by the thiosulfate-oxidizing enzyme. The reaction mixture contained 50 mM sodium-phosphate buffer, pH 6.1, 11 μM HiPIP, 20 μg enzyme, and thiosulfate at the concentrations indicated.

Table I. Reactivity with various electron acceptors of thiosul-
fate-oxidizing enzyme. Experimental conditions were as described
in the legend for Figure 4.

Electron acceptor	Electron acceptor reduced (nmoles/min)	
	10 mM phosphate	maximum
Chromatium HiPIP	4.04	10.3
Chromatium cytochrome c-552	0	0
Chromatium cytochrome c-553	0	0
Chromatium cytochrome c'	0	0
Yeast cytochrome c	5.62	5.62
Tuna cytochrome c	7.07	10.2
Horse cytochrome c	0.560	0.560

Table II. Effects of sulfite, cyanide, and heating on the reduc-
tion of HiPIP catalyzed by thiosulfate-oxidizing enzyme with
$Na_2S_2O_3$. The reaction mixture contained 50 mM sodium phosphate
buffer, pH 6.1, 3.3 mM $Na_2S_2O_3$, and 20 µg enzyme preparation.

Conditions	HiPIP reduced (nmoles/min)
Complete	7.6
+KCN (1.3 mM)	3.2
(2.0 mM)	2.2
+SO_3^{2-} (1.7 mM)	3.6
Heated ($100^{\circ}C$, 1 min)	0

Table III. Stoichiometry in reduction of ferricyanide catalyzed
by C.vinosum thiosulfate-oxidizing enzyme in the presence of thio-
sulfate. The concentration of ferricyanide was 1.5 mM, while
that of thiosulfate was maxmally 0.67 mM. A total volume of the
reaction mixture was 3.0 ml.

Experiment	$Na_2S_2O_3$ added (μmole)	$K_3Fe(CN)_6$ reduced (μmole)
1	0.400	0.430
2	0.800	0.810
3	1.20	1.20
4	1.60	1.58
5	2.00	1.98

This cytochrome is thought to correspond to Chlorobium cytochrome c-555 in functional sense (2,10). However, the Chromatium enzyme utilizes HiPIP as the electron acceptor. The structure of HiPIP isolated from C.vinosum have been extensively studied (15,16). However, its physiological role was not found although its participation in the photosynthetic electron transfer of the organism has been suggested (17). In the present work we have shown that the protein acts as the electron acceptor for the thiosulfate-oxidizing enzyme. HiPIP has been purified mainly from purple photosynthetic bacteria (18). As nonsulfur purple bacteria do not utilize thiosulfate as the photosynthetic electron donor, it may be that the function of HiPIP as the electron acceptor for the thiosulfate-oxidizing enzyme is not sole physiological role of the protein. In any case, it may be said that the present investigation has revealed at least one of the functions of the protein.

As the oxidation product of thiosulfate by the enzyme seems to be tetrathionate, it may be concluded that the present work has also confirmed the results obtained by Smith (8) with partially purified enzyme and native electron acceptor. Chromatium vinosum accumulates elementary sulfur in the cells even when the organism was cultivated in the presence of thiosulfate but in the absence of sulfide. Therefore, the organism should possess an enzyme system which produces elementary sulfur from thiosulfate directly or via tetrathionate. If elemental sulfur is produced directly from thiosulfate, the organism should have another enzyme which participates in oxidation of thiosulfate. Thus, it has been reported that an enzyme with the rhodanese activity exists in the organism.

However, in the present investigation, the rhodanese activity
has not been detected with the cell free extract from the organism.
If elemental sulfur is produced via tetrathionate, the thiosulfate-
oxidizing enzyme obtained in the present investigation can be a
main enzyme in the oxidation of thiosulfate in the organism.
In any case, it has been found in the present investigation that
HiPIP can participate in oxidation of thiosulfate in Chromatium
vinosum.

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